Diagnostics and molecular epidemiology of the *Sarcoptes scabiei* mite infesting Australian wildlife

Tamieka Fraser

B. AppSc (1st Class Honours)

September 2018

Submitted for the degree of Doctor of Philosophy to the Department of Biological Sciences, University of Tasmania and the Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast

This thesis is dedicated to my amazing parents, Dean and Suellyn Fraser, who have always encouraged me to never give up in the face of hard times and inspire me every day to have a positive attitude on life.

APPROVALS

Doctor of Philosophy Dissertation

Sarcoptic Mange in Australian Marsupials

By Tamieka Fraser, B. AppSc (Hons)

Supervisor: _____

Dr. Scott Carver

Co-Supervisor: _____

Professor Adam Polkinghorne

STATEMENT OF ORIGINALITY

I declare that this is my own work and has not been submitted for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others have been duly acknowledged in the text and within the reference list.

	08 September 2018
(signature)	(date)

STATEMENT OF ACCESS

This thesis may be made available for loan and limited copying in accordance with the *Copyright Act 1968*

	08 September 2018
(signature)	(date)

STATEMENT REGARDING PUBLISHED WORK

The publishers of the papers comprising Chapters 2, 3 and 5 hold the copyright for that content, and access to the material should be sought from the respective journals. The remaining non-published content of the thesis may be made available for loan and limited copying and communication in accordance with the above Statement of Access and the *Copyright Act 1968*.

Due to the inclusion of published material there is unavoidable repetition of material between chapters in this thesis.

	08 September 2018
(signature)	(date)

STATEMENT OF ETHICAL CONDUCT

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

	08 September 2018
(signature)	(date)

ACKNOWLEDGEMENTS

First must go to my two supervisors. Scott Carver, thank you for taking me on and putting up with my stubbornness. Your patience, guidance and continual support has been tremendous. For always knowing exactly what to say when I second guessed myself and my abilities, for helping me expand the way I think and write, and the fantastic field work sessions; it has been a wonderful experience. Adam Polkinghorne, it has been a long journey since I started my research career with you back as a young sprightly honours student. Thank you for always challenging me, I know my research career would have been very different if it hadn't been for your assistance and guidance. Your encouraging words of "to see each day as a new day" has truly spurred the successful completion of my thesis.

With two great leaders comes two remarkable research groups. To the "Carver Lab", all wonderful lab mates, constant array of laughter and warm welcoming, you made Hobart feel like a second home. Special mention must go out to Alynn Martin, my fellow wombat warrior and the queen of caramel slice. This PhD would not have been anywhere near as amazing without you fighting alongside me. I am so bloody proud of you and cannot wait to follow your research career; you are going to smash it! To the #Chlamydiansforlife past and present members, you all have been a definitive influence in my life one way or another. Thank you all for pushing me to always strive for perfection and constantly supporting me. Most importantly, Martina Jelocnik; from being my demonstrator in undergrad to my mentor in honours and PhD, not only do I have the uttermost respect for you, I admire your forever remembering brain, your loving and compassionate heart.

I would not have been able to make it through this past eight years if it hadn't been for some legends! To Becky Moore, the sister I choose, there will never be enough words to express my uttermost love and gratitude for you. You are my person and your undying support in every

aspect of my life has been a godsend. To Danielle Madden, I am so grateful that I met you and convinced you to leave freezing Hobart to follow me up to the Sunshine State. Thank you for always being my biggest advocate and drinking buddy. I can't wait to see you flourish as you progress through your PhD. To Rachel Self and Anna Chruścik, I can't believe we started together in undergrad and are all now towards the end of our PhDs. It has been a wonderful exciting journey with you both. To Jess Levick, thank you for being an amazing friend and personal cheerleader! To Erin Price and Derek Sarovich, thank you for all the laughter, you guys are fabulous! To Delaney Burnard; it's been amazing going through this journey together.

Most importantly, I have my wonderful family to thank. Firstly, to my amazing parents Dean and Suellyn, without you both pushing me to finish, supporting and encouraging me when I was struggling, celebrating both the big and small achievements and nodding along when I rambled on about my research, I do not think I would have achieved half of what I have. To my little sister Makalah, thank you for being my shoulder to cry on and my person to whinge to and practice my knowledge and talks on. To my amazing grandparents, thank you for always believing in me and for the encouragement you have given me throughout my studies. My family is the reason I do anything, I love you all.

And lastly, but certainly not least, to my wonderful partner John Nugent. Starting a relationship while I was in Tassie as long distance, putting up with my stress and the continuous ups and downs that come with research could not have been easy. Thank you for being my rock and talking some positive sense into me when I didn't believe in myself.

I would also like to acknowledge Holsworth Wildlife Research Endowment for supporting this research.

PUBLICATIONS ARISING FROM THIS THESIS

The following chapters consist of publications that have arisen from this thesis;

CHAPTER 2: **Fraser TA**, Charleston M, Martin A, Polkinghorne A, Carver S: The emergence of sarcoptic mange in Australian wildlife: an unresolved debate. Parasites & Vectors 2016, 9(1):1-11.

CHAPTER 3: **Fraser TA**, Shao R, Fountain-Jones NM, Charleston M, Martin A, Whiteley P, Holme R, Carver S, Polkinghorne A: Mitochondrial genome sequencing reveals potential origins of the scabies mite *Sarcoptes scabiei* infesting two iconic Australian marsupials. BMC Evolutionary Biology 2017, 17(1):233.

CHAPTER 5: **Fraser TA**, Martin A, Polkinghorne A, Carver S: Comparative diagnostics reveals PCR assays on skin scrapings is the most reliable method to detect *Sarcoptes scabiei* infestations. Veterinary Parasitology 2018, 251:119-124.

CHAPTER 6: **Fraser TA**, Carver S, Martin AM, Mounsey K, Polkinghorne A, Jelocnik M: A *Sarcoptes scabiei* specific isothermal amplification assay for detection of this important ectoparasite. PeerJ 2018, 6:e5291.

The following chapter consist of an accepted manuscript for peer reviewed publication:

CHAPTER 4: **Fraser TA**, Holme R, Martin AM, Whiteley P, Montarello M, Raw C, Carver S, Polkinghorne A: Expanded molecular typing of *S. scabiei* provides further evidence of disease spill-over events in the epidemiology of sarcoptic mange in Australian marsupials. Journal of Wildlife Diseases, Accepted 13th June 2018.

THESIS STRUCTURE

In total, this thesis contains 7 chapters and is structured as follows;

- Chapter 1 is a general introduction to the parasite studied, *Sarcoptes scabiei*, the disease it causes (sarcoptic mange) and the problematic diagnosis and lack of comprehensive genetic tools for studying the epidemiology of this mite.
- Chapter 2 is a critical review on the gene targets previously used for molecular typing of *S. scabiei* and highlights the variability and inconsistencies from a reanalysis of globally available sequences.
- Chapter 3 characterises the first mitochondrial genomes of koala and wombat derived *S. scabiei* mites and used comparative analysis to determine which gene/s should be used for subsequent molecular typing investigations.
- Chapter 4 expands on the analysis of Australian-derived mites and, using the gene identified by Chapter 3 for molecular typing analysis, reveals genetic evidence for cross-host transmission of *S. scabiei* between marsupial species and from domesticated animals in Australia but also those found globally.
- Chapter 5 describes a comparative assessment of currently used clinical and veterinary diagnostic procedures for *S. scabiei* and expands this analysis to include a non-invasive method and an alternative molecular technique.
- Chapter 6 describes the first isothermal nucleic acid amplification assay for detection of *S. scabiei* DNA that has direct implications to rapid detection of this mite in clinical specimens in field and clinical settings.
- Chapter 7 is a summary of the findings and significance of this research to existing literature with implications for future research.

TABLE OF CONTENTS

THESIS ABSTRACT

Parasitic infestations have always been a noteworthy topic for human and animal health globally, with many considered a result of spill-over and zoonosis. One such parasite, *Sarcoptes scabiei*, is known to infest over 300 million humans per year and has been documented in over 104 mammals. It has recently been classified as a neglected tropical disease and is among the top 50 most prevalent human diseases. With uncertainty over (i) the global epidemiology of *S. scabiei* and (ii) the reliability of current diagnostics methods, much still needs to be understood if stakeholders are to successfully develop strategies to control this parasite.

The overall objective of this thesis was to study the molecular epidemiology and genetic typing of *S. scabiei* infesting Australian animals and assess the diagnostic methods for sarcoptic mange. At the global scale, numerous genetic studies have attempted to reveal how the host species and host geographic location influence *S. scabiei* phylogenetics. By performing an analysis of the global literature (Chapter 2), I was able to reveal that there were inconsistencies in gene loci and phylogenetic conclusions used in these previous studies. Furthermore, by executing a contemporary analytical approach employing molecular markers on existing *S. scabiei* sequences, it was apparent that (i) new *S. scabiei* samples, (ii) appropriate gene loci targets, and (iii) advanced phylogenetic approaches are necessary to more confidently comprehend the origins of mange in Australia.

As there were only a limited number of Australian marsupial-derived *S. scabiei* sequences, and that three of the most commonly used gene loci used for typing are located within the mitochondria, I performed mitochondrial genome sequencing of mites collected from koalas and wombats (Chapter 3). It was revealed that there is a high sequence similarity not just within marsupial *S. scabiei* mites, but also to the only human-derived *S. scabiei* mitochondrial genome.

Furthermore, by examining individual gene phylogenies, I concluded that *cox1* is the most informative gene as the *cox1* phylogeny inferred was consistent with the complete mitochondrial genome phylogeny with the highest resolution of ancestral lineages.

Building on the identification of *cox1* as an informative gene target, I greatly expanded the molecular typing of *S. scabiei* within Australia (Chapter 4). I identified that mites collected from koalas, wombats, foxes and dogs across five states of Australia were unable to be phylogenetically separated by their host or location. Thus, I considered it highly plausible that multiple spill-over events may have occurred in Australia, as many haplotypes are identical to European and non-European sequences. Furthermore, I suggested that it is likely that canids are the source for transmission of mange throughout Australian wildlife as dogs and foxes share identical haplotypes to wombats and koalas. Finally, I detected a distinguishable human-specific lineage, distinct from the dominant mixed animal clade.

Clinical diagnosis of mange/scabies typically involves the collection of skin scrapings followed by microscopic detection of the mite. This method yields results with a high risk of false negatives, however. I performed the first comparative *S. scabiei* diagnostic study on a unique sample set collected from bare-nosed wombats. Here, I assessed a variety of putatively useful approaches including observational scoring, microscopy, PCR on skin scraping DNA and PCR on skin swab DNA (Chapter 5). I concluded that: (i) observational scoring positively correlated with counts from microscopy, however this approach tended to under-diagnose early mange; (ii) species-specific *S. scabiei* PCR enhanced the sensitivity of mite detection in relation to microscopy and; (iii) swabs as a method for sample collection is questionable due to inadequate host cell uptake and likelihood of producing false negatives.

Finally, I sought to improve the use of molecular techniques for *S. scabiei* diagnosis (Chapter 6). I developed a novel rapid diagnostic tool using a Loop Mediated Isothermal Amplification

assay, which I demonstrated to be specific to *S. scabiei* and able to produce a rapid diagnostic result within 30 minutes. Since this method can be performed without advanced laboratory equipment, this development has potential direct roles as an ancillary method with microscopy at the point-of-care to reduce the number of potential *S. scabiei* false-negative results obtained by microscopy alone in both human and veterinary settings.

In summary, this thesis has contributed to: (i) the expansion of *S. scabiei* phylogeny by highlighting the high genetic variability of the single mite species; (ii) suggested multiple spill-over events may be the consequence of inadequate screening of imports/exports possibly globally and; (iii) has demonstrated the incompatible variety of different diagnostic methods for *S. scabiei* which may be supplemented with the development of a new highly sensitive and specific molecular technique. The contributions I have made in *S. scabiei* research will aid in future conservation efforts to aid in understanding transmission risks to threatened populations and enhance diagnostic procedures in clinical, field and remote settings.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 A history of pathogen spill-over proves to be an ongoing burden

The "One Health" triad encompasses human and animal health, and their environmental contexts, as a concept for general wellbeing, advancement in clinical and veterinary care and enhanced public health efficacy [1, 2]. A growing research focus in this initiative targets the drivers and epidemiology of zoonosis and pathogen spill-over in the context of controlling emerging infectious diseases in humans and animals. Zoonotic diseases have global implications as they encompass 58% of the estimated 1407 species of recognised human pathogens [3]. Human activity and movement has been linked to pathogen spill-over events for many infectious agents, either by humans themselves or by their companion and agricultural animals [4, 5]. As a result, epidemic health risks and population loss to naïve host species has occurred [6].

Pathogen spill-over from a reservoir host to a naïve wildlife host has proven to be problematic for wildlife conservation. Canine distemper virus is a prime example of dissemination and genetic spill-over from dogs to wildlife, causing mortality in a variety of animal hosts [7-10]. Australia is known for its unique wildlife. Despite this, since European arrival, a number of the nation's iconic species are now under the threat of extinction. Introduced pathogens, among threats from habit destruction and invasive predators, are recognised as one of the major causes of wildlife decline in Australia. *Chlamydia pecorum* is a prime example of a sourced pathogen spill-over event that saw imported European livestock transfer *C. pecorum* to Australian marsupials, directly contributing to the decline of naïve koala populations [11]. *Sarcoptes scabiei* is an emblematic pathogen for pathogen spill-over and wildlife population declines, both globally and within Australia.

1.2 Biology of *Sarcoptes scabiei*

The subclass Acari, containing mites and ticks, is estimated to contain between 0.5 to 1 million species of which only approximately 5% are described [12]. Many are classified as agricultural pests and/or of medical and veterinary concerns due to their role in predatory plant feeding, asthma associated allergies, diseases in livestock causing economic loss, native animal population collapse, and human socioeconomic disease burden [13]. The "itch" mite, *S. scabiei* is a single species of the *Sarcoptes* genus under the family Sarcoptidae [14]. Within this family, *Sarcoptes* and *Trixacarus* are the most morphologically similar, however key features including size and host speciation can be visually and conclusively distinctive [14, 15]. There are five developmental stages of *S. scabiei* consisting of egg, larva, protonymph, trionymph and adult, with the developmental cycle reported to fall within seven to 21 days [14, 16, 17]. Female mites are documented to lay two to four eggs per day and adult mites emerge on the surface of the hosts skin within 14 days [18, 19].

1.3 Disease pathology for sarcoptic mange

S. scabiei is known to infest over 104 species of mammals, and cause significant economic burden and health and welfare concerns for both humans and animals [20]. Sarcoptic mange/scabies can be classified into two diseases states based on the presentation of clinical symptoms; ordinary scabies and crusted scabies. Ordinary scabies is associated with low mite burden (approximately 10 to 15 mites per person) with clinical symptoms including erythematous papules, allergic skin reactions accompanying with intense and generalised pruritus [21]. Crusted scabies is considered more contagious due to the thousands of mites infesting an individual and the subsequent development of hyperkeratosis/parakeratosis, emaciation, severe alopecia and painful skin fissures [22]. Both ordinary and crusted scabies patients are susceptible to secondary bacterial infections of exposed skin lesions, the result of

severe itching, by *Staphylococcus aureus* and *Streptococcus pyogenes*, however this is more commonly seen with crusted scabies infestations [22]. Sepsis, kidney and heart disease and lymphadenopathy can develop if left untreated and may lead to death [20, 23]. Other than the clinical manifestations, the immune response to crusted and ordinary scabies also differs. A non-protective Th2 allergic response is associated with crusted scabies whereas ordinary scabies establishes a cell-mediated protective Th1 response [24]. Like many other parasites [25, 26], manipulation of the host's immune response to establish an eventful manifestation has been recognized for *S. scabiei* [14]. The suppression of early infestation symptoms appears to result from secretory antigens released from the mite into the intercellular fluid of the epidermis [14, 27].

Cutaneous hypersensitivity associated with ectoparasite infestation has been associated with a variety of parasites including *Chorioptes bovis* in horses [28], *Psoroptes ovis* in cattle [29] and *Demodex folliculorum* in humans [30]. Clinical symptoms of severe itching of scabies has been reported to develop within four to eight weeks of primary infestations in humans [31]. This delay has been associated to a hypersensitivity reaction, suspected to be the result of the mite developing the capability of modulating the immune response of its host [22, 27]. However, subsequent reinfestations can be identified within 24-48 hours and is presumed the patient has been desensitised with past infestations and accumulated immunological antigen memory [24, 31, 32]. Experimental mite infestations of low and high doses in pigs identified that, for high doses, individuals developed hypersensitivity faster than low doses [33]. Erythema is the first clinical sign to develop in wombats and has been reported to arise within 14 days of infestation, and within 24 hours after reinfestation, suggestive of hypersensitivity as a factor in the pathogenesis of mange in this marsupial [34, 35].

1.4 Diagnosis of *S. scabiei*

Typical diagnosis of sarcoptic infestations include observation of clinical signs, coupled with microscopy confirmation of eggs and/or mites within a skin scraping of the suspected area [36]. In wildlife medicine, field observations have been utilised for wolves, coyotes, chamois and wombats to 1) limit the degree of excessive handling and stress upon the animal and 2) is a cost efficient and simpler method, rather than by live capture, to obtain an epidemiologically representative number of free-ranging individuals [37-41]. However, these methods of diagnosis have proven to be problematic, particularly for the diagnosis of early infestation that corresponds with low mite burden and hypersensitivity delaying symptom progression. For example, infestations of the red fox and coyote can develop severe disease (usually ordinary scabies/mange) with very low mite burden, limiting the successful use of microscopy alone as a method for detection of mites in the absence of obvious clinical signs [20, 42, 43]. Clinical manifestations further complicate diagnosis as ordinary scabies and early crusted scabies symptoms can mimic insect bites, eczema, diaper dash, psoriasis, dermatitis and irritants from soaps, latex and other chemicals [14].

With evidence continuing to grow about the host expansion of *S. scabiei*, the accurate diagnosis of early stage mite infestation in naïve hosts has become more important than ever. To improve the identification of early infestations of scabies/mange, a range of *S. scabiei* antigen-based assays have been investigated [44]. Native mite antigens have been suggested to be an ideal target, however are far from optimal in regards with both sensitivity and specificity [14]. However, specific antibodies have been revealed in as little as 15 days post experimental infestations, either before or simultaneous to appearance of first mild lesions in red fox [45, 46] and Spanish Ibex [47]. Although these sensitivity and specificity concerns are present, future sero-diagnosis used in parallel with molecular techniques could remedy this. Additionally there is high cross-reactivity between *S. scabiei* and house dust mites, producing inconclusive

outcomes for whether antigens can be used as a serological test [48]. Many other studies have attempted to develop an ELISA assay for both humans and animals, but again demonstrate varying sensitivity and specificity with either monoclonal antibodies [45, 49-52] or recombinant molecules [53-56].

1.5 Off-host parasite survival plays a significant role in mite transmission

Infectious agents are transmitted between individuals by a number of means including direct contact, sharing living space, sexual relations and consumption, however the relative role of each of these methods is dependent on their infectious form and ability to survive off its host. Direct and environmental transmission are important factors for *S. scabiei*. In humans, scabies transmission has been suggested to predominate via direct contact rather than sharing habitual space with infested people [14]. A large study consisting of 272 people found that only 0.01% (4/272) of the subjects sharing beds with infested individuals developed scabies [16]. Mite survival off the host has been investigated primarily surrounding temperature and humidity. *S. scabiei* in dogs have been documented to survive off its host for over a week at relative humidity above 75% and low temperature (15°C), with survival decreasing significantly with warmer temperatures as a result of dehydration [57]. For animals it has been suggested that dens or burrows are a common ground for indirect transmission, owing to low temperatures and high humidity in the environment and several burrowing and den using species commonly contracting *S. scabiei* infestations [58, 59].

1.6 Cross-infestations and genetic similarity of variants for *S. scabiei*

Attempts to clarify if cross-host species infestations do indeed occur have been documented with varying results of self-limiting and successful cross-infestation experiments. *S. scabiei* var *canis* mites have been known to establish experimental infestation on rabbits, guinea pigs, sheep, calves and humans [49]. However, experimental infestations of pigs with var *suis* mites

have shown to have mixed outcomes of both established infestation and self-limiting cases [60]. Human infestations contracted from animals have reportedly been restricted to certain topographies and are largely self-limiting [61]. Zoonotic transmissions to humans, generally by dogs, are usually less severe and short lived estimated to occur up to 50% of the time [62-66].

Is has been suggested that humans and protohumans are the initial source of mange, with subsequent adaptation to other animal hosts [67]. Attempts to genetically answer if mites are host selective include whole genome sequencing and single gene molecular typing. There is only a single annotated draft genome for *S. scabiei* var. *canis* [68], and available genomic resources and draft assemblies for *S. scabiei* var. *hominis* and var. *suis* [69] are genetically very similar. Depending on the gene target used (ITS-2, 16S rRNA, 12S rRNA, *cox1* and microsatellites) it has been proposed that *S. scabiei* can be differentiated by either host or location [67, 70-73]. A dearth of genetic resources for this species otherwise makes studies of the genetic relationships of *S. scabiei* and hence an understanding of the epidemiology of this pathogen challenging.

1.7 Sarcoptic mange within Australian wildlife

There is a high prevalence of sarcoptic mange in Australia, particularly in Aboriginal communities and certain native wildlife populations [19]. Although known to sporadically infest the koala [74], agile wallaby [75], swamp wallaby [76], southern brown bandicoot [77] and dingo [78, 79], mange is known to cause severe pathology in two species of wombat; the bare-nosed and southern hairy-nosed [59, 80]. Southern bare-nosed wombat populations, in particular those located in New South Wales, Victoria and Tasmania, have experienced isolated population declines of up to 95% [38, 58]. While considerable research has been undertaken investigating the pathology and ecology of wombat mange [40, 81], there has been limited

research on how mange was introduced into Australia. With the assumption that European settlers and their dogs were the source, only historical documentation and two more recent genetic studies have investigated this theory [58, 59, 72, 82]. In the absence of this information, it is currently unknown why mange appears to emerge sporadically in presumably naïve marsupial populations across the country.

1.8 Aims of this thesis

The purpose of this thesis is to explore the knowledge gaps surrounding the introduction, incidence and spill-over of *S. scabiei* in Australian marsupials and evaluate the current diagnostic methods used in field and clinical settings. To address the questions raised surrounding *S. scabiei* and the overall objective of this thesis, the research described in this thesis was designed to achieve the following aims to:

1. Evaluate the current molecular markers used for *S. scabiei* and review the existing Australian-derived sequences
2. Establish a population-genetic framework for *S. scabiei* in Australian marsupials using the first mitochondrial genome sequences of animal-derived mites
3. Expand the population-genetic framework of Australian derived *S. scabiei* mites and assess the inter- and intra-population genetic diversity within Australia and globally
4. Assess the current methods used in field and clinical diagnosis of mange
5. Determine if nucleic acid detection could improve diagnosis or be used as an ancillary method for detection of *S. scabiei*

1.9 Project sample collection

The host species used in this thesis includes wombats, koalas, foxes and domestic dogs, however the majority of samples collected were obtained from bare-nosed wombats from Tasmania and New South Wales. The access to wombats presenting to wildlife hospitals in several Australian states with a spectrum of *S. scabiei* disease from early infestation to severely mangy wombats, not only enabled analysis of the genetic diversity of geographically distinct populations but also supported the use of the wombat as a model for assessing different diagnostic approaches to *S. scabiei* detection.

Koala mange is also becoming more prevalent, particularly in the southern states of Australia, which meant that these samples collected from the Victorian and South Australian koalas were vital in confirming the etiological agent, *S. scabiei*, but also to expand epidemiological studies of this mite into sympatric populations of other marsupials. Additionally, the samples collected from foxes and dogs allowed for the assessment of transmission between canids and native marsupials.

CHAPTER 2

THE EMERGENCE OF SARCOPTIC MANGE IN AUSTRALIAN WILDLIFE: AN UNRESOLVED DEBATE

Tamieka A. Fraser[1,2], Michael Charleston[1,3], Alynn Martin[1], Adam Polkinghorne[2] and Scott Carver[1]

[1] School of Biological Sciences, University of Tasmania, Sandy Bay 7001, TAS, Australia.

[2] Centre for Animal Health Innovation, Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, 91 Sippy Downs Drive, Sippy Downs 4556, QLD, Australia.

[3] School of Information Technologies, University of Sydney, Camperdown 2006, Sydney, Australia.

This paper was published in BMC Genomics, 2016. 9(1): p. 1-11

Statement of joint authorship

TAF, MC, AM, AP, SC equally conceptualised the topic and development of this review. TAF wrote this manuscript with drafting contributions and approval from remaining authors. Network tree analysis was completed by TAF and MC. All authors read and approved the final version of the manuscript.

2.1 Abstract

Due to its suspected increase in host range and subsequent global diversification, *Sarcoptes scabiei* has important implications at a global scale for wildlife conservation and animal and human health. The introduction of this pathogen into new locations and hosts has been shown to produce high morbidity and mortality, a situation observed recently in Australian and North American wildlife.

Of the seven native animal species in Australia known to be infested by *S. scabiei*, the bare-nosed wombat (*Vombatus ursinus*) suffers the greatest with significant population declines having been observed in New South Wales and Tasmania. The origins of sarcoptic mange in Australian native animals are poorly understood, with the most consistent conclusion being that mange was introduced by settlers and their dogs and subsequently becoming a major burden to native wildlife. Four studies exist addressing the origins of mange in Australia, but all Australian *S. scabiei* samples derive from only two of these studies. This review highlights this paucity of phylogenetic knowledge of *S. scabiei* within Australia, and suggests further research is needed to confidently determine the origin, or multiple origins, of this parasite.

At the global scale, numerous genetic studies have attempted to reveal how the host species and host geographic location influence *S. scabiei* phylogenetics. This review includes an analysis of the global literature, revealing that inconsistent use of gene loci across studies significantly influences phylogenetic inference. Furthermore, by performing a contemporary analytical approach on existing data, it is apparent that (i) new *S. scabiei* samples, (ii) appropriate gene loci targets, and (iii) advanced phylogenetic approaches are necessary to more confidently comprehend the origins of mange in Australia. Advancing this field of research will aid in understanding the mechanisms of spill-over for mange and other parasites globally.

Keywords: *Sarcoptes scabiei*, Wombat, Network, Phylogeny, One Health, Conservation Medicine

2.2 Background

The spread of pathogens from endemic to novel host foci, otherwise known as spill-over, is one of the most significant threats to the health of both animals and humans, globally [5, 83]. Approximately 61 % of known human pathogens are zoonotic and up to 90 % of pathogens infecting animals are transferable between other animal species [84]. Identification of spill-over reservoirs is important for management attempts to intervene in further pathogen pollution and determining if long established diseases are invasive. Indeed the latter of these can be critical for justifying management, particularly for establishing whether a pathogen is native" or invasive to a host or region and if it warrants control in wildlife. Modern molecular techniques, including phylogenetic comparisons and metagenomics, have revolutionised our ability to identify spill-over and characterise pathogens [85, 86]. In this review, we focus on an important example of disentangling the origins of a pathogen causing significant disease burden. Sarcoptic mange (causative agent *Sarcoptes scabiei*) is a major disease of Australian wildlife, particularly to wombats (bare-nosed/common and southern hairy-nosed), and also impacts humans, domestic animals, and other Australian wildlife, with negative economic outcomes [87]. *Sarcoptes scabiei* is a parasitic astigmatid ectoparasite which feeds off skin cells and serum as it burrows into the epidermal and dermal layer of its host. It has an extremely wide host range, infecting over 104 mammal species, and is a global contributor to the world's burden of parasitic infestations [20]. Similar to what has been seen for emerging viral diseases, this mite has had an important role in shaping host populations, causing the collapse of several host species around the world [88]. *Sarcoptes scabiei* is known to infest both humans and animals; in the former, the resulting disease is referred to as scabies, whereas infestation of domesticated animals and wildlife is referred to as mange [19]. The broad host range of

sarcoptic mange commonly includes domestic dogs, livestock (e.g. cattle, pigs, goats, camelids) and wildlife (e.g. red foxes, coyotes, wolves, deer, bobcats, wombats, koalas and wallabies) and poses an important welfare and economic burden, globally [87, 89]. More recently the need for greater research on this largely neglected pathogen has been highlighted owing to its resurgence and emergence in several areas across the globe [90, 91], leading to its classification as a wildlife emerging infectious disease [5], particularly owing to host range expansion in Australia and North America.

The origins and even endemicity of this pathogen have been the source of much debate. Here, we review the genetic evidence of host specificity and cross-species transmission of *S. scabiei*, examine the strengths and limitations of the existing literature around this topic, and propose critical directions for more clear and concise answers into the degree of variation (information) provided by genetic data. We focus on Australia as a case study, owing to the importance of this pathogen at a national scale, particularly in wildlife conservation and human and domestic animal health. However, the broader principles apply to mange in many other regions globally, (e.g. North America) and other pathogens with debated origins (e.g. Chlamydia in koalas).

2.3 Historical origins and epidemiology of mange in Australian wildlife

Questions over the origin, reservoirs and transmission of *S. scabiei* mites in and between Australian wildlife host species have been ongoing for nearly two centuries [59]. These questions have persisted owing to their perceived importance for detaining the reservoirs of infections and controlling this pathogen. General perceptions have been that mange was introduced into Australia by European settlers and/or their domestic dogs [59]. Mange is known to affect a number of Australian wildlife species including the koala [92], agile wallaby [75], swamp wallaby [76], southern brown bandicoot [77], dingo [78, 79] and the bare-nosed and southern hairy-nosed wombat [59]. The earliest records of mange on an Australian animal date

back to Latreille (1818), where mites infecting a wombat held at the Muséum national d'Histoire naturelle in Paris, were identified as identical to *S. scabiei* found on a human male, however it is possible that mange was contracted in translocation [59]. It was not until 1937 that mange was first identified in a New South Wales bare-nosed wombat population which had undergone a large population decline, most likely due to the disease [58].

There has been considerable debate and anecdotal evidence surrounding the role that foxes and wild dogs may have in the transmission of mange to Australian wildlife [93]. The red fox was introduced into Australia in 1850 and is known to be a host to *S. scabiei* [78]. Since (i) mites are capable of surviving in low temperatures and high relative humidity for extended periods of time of potentially up to three weeks [32, 94], and (ii) it has been documented that canids periodically enter wombat burrows, it is possible that the route for transmission between both canids and wombats occurs via burrows [59]. Furthermore, domestic dogs have been shown to contract mange after predating upon mangy wombats [58]. Some suggestions have been made that canids may be necessary for disease persistence in marsupials [34, 95]. In contrast to these hypotheses, persistent disease is observed in Tasmanian bare-nosed wombats where foxes are considered absent [96]. Thus, evidence suggests mange can persist in Australian wombats, and possibly other wildlife, with or without the involvement of canids. *Sarcoptes scabiei* infestations have also been widely reported in Australian indigenous communities, domestic dogs and livestock, with ongoing economic costs associated with human health and animal health and production [87, 97]. Extensive studies of this ectoparasite in humans, pigs and dogs have recently been performed with an emphasis on developing a vaccine [60, 87, 98-100].

2.4 Prevalence and pathology of sarcoptic mange in Australian wildlife

The pathology associated with mange in Australian marsupials is consistent with other animals globally, suggesting the symptomology is not unique to Australia (and by extension the mite,

or strain of mite, is also not distinct). Symptoms include irritation, inflammation, hyperkeratosis, alopecia, pruritis, dermatitis and lesions that are typically coupled with pneumonia and secondary infections [20, 101]. Although the koala, wallaby, possum, bandicoot and wombat all have shown signs of mange, the most severe pathology and consequent conservation threat is to the bare-nosed and southern hairy nosed wombats, as mange has been shown to cause localised extinction in isolated populations [34]. Of the three species of wombat living in Australia, the bare-nosed wombat appears to be more susceptible, suffering higher morbidity and mortality [34]. For example, mass declines of 70 % in bare-nosed wombat populations in New South Wales [58] and > 80 % in a bare-nosed population in Tasmania [38, 40], have been documented. It is likely that numerous other population declines have occurred but gone undocumented owing to the absence of reliable information on the prevalence and distribution of individual wombat populations. However, it is also notable that other populations of bare-nosed wombats may experience more variable impacts, such as endemic dynamics with low background mortality. The severity of this disease impacts on wombats, and the ability of this pathogen to drive catastrophic declines, have contributed to the classification of this pathogen to likely be an introduced pathogen to wombats, and Australian wildlife more broadly, and have also spurred periodic genetic studies to address this.

2.5 Genetic attempts to identify the origins of mange in Australian wildlife

To date, attempts to answer questions concerning the origin of mange in Australian wildlife have largely centred on the use of individual genetic markers to identify similar, if not genetically identical, mites between wildlife and humans in Europe, Asia and Australia. Skerratt et al. [82] identified mites from wombats, dogs and humans in Australia to have similar 12S rRNA gene sequences and concluded that European settlers and their domestic dogs introduced mange into Australian wildlife. Following this study, Walton et al. [72] expanded not only the known host range of mange in Australia but targeted three different gene regions

of *S. scabiei* for genetic comparisons: cytochrome c oxidase subunit I (COX1), 16S rRNA gene and microsatellites. Analysing microsatellites and COX1 sequences, Walton et al. [72] revealed that wombat *S. scabiei* sequences separated into their own subclade within a human and animal clade (dog, human, chimp, wallaby, wombat and fox). However, conflicting results occurred for the wombat sample when evaluating the 16S rRNA gene sequences, as the mite extracted from the wombat was identical to a canine *S. scabiei* 16S rRNA gene sequence [72]. More recently two studies [67, 102] based on data obtained from *S. scabiei* from France concluded that *S. scabiei* was introduced into Australia by European settlers based on a single French human *S. scabiei* sequence being identical to the reference *S. scabiei* var *wombatii* by 12S rRNA gene, and clustering of French and Australian human mites based upon COX1 sequences. An obvious limiting factor in these studies is the lack of new Australian samples used to accurately confirm their conclusions, as the 12S rRNA gene and COX1 sequences used were originally obtained by Skerratt et al. [82] and Walton et al. [72].

Of these four Australian marsupial studies [67, 72, 82, 102], spanning nearly 15 years, it is notable that their conclusions have been strongly influenced by the choice of molecular marker gene and the geographical locations of both animal and human mite samples. Two of these studies [82, 102] have used the 12S rRNA gene, but is it increasingly recognised that this locus is relatively uninformative of phylogenetic structure among host species and populations for *S. scabiei* [72, 103, 104]. Contrastingly, gene loci COX1, 16S rRNA and microsatellites, used by Walton et al. [72] had greater genetic discrimination and, accordingly, the authors identified significant genetic structure based upon host species and geographical location. Additionally, the COX1 gene contains numerous sites where single nucleotide polymorphisms can occur in this relatively conserved part of the mitochondrial genome, with a mutation rate rapid enough to distinguish between closely related species [72, 105]. Although microsatellites have the potential to support investigations of genetic structure of natural populations where

environmental barriers, mating systems and historical processes can alter the genetic structure [106], Walton et al. [72] clearly revealed higher genetic discrimination using COX1 compared to their microsatellite results. Furthermore Walton et al. [72] was able to demonstrate significant relationships between their 16S rRNA gene haplotypes, similar to COX1, which is interesting since the use of 16S rRNA is similar to 12S rRNA, as it is valuable for the identification of species but limited for intra-species analysis [107].

Beyond the choice of gene markers for studying the genetic relationships of mites from different hosts, the simple fact is that adequate sampling is still a major limitation to answering questions of this nature. Collectively, the mites used for these Australian *S. scabiei* sequence comparison studies include a total of eight wombats from Victoria, one wombat from South Australia, 17 humans and ten dogs from the Northern Territory, along with samples from outside of Australia, including: ten humans from Panama, ten dogs from the USA, one chimpanzee from Tanzania, one fox from Sweden, and two dogs, one pig and 83 humans from France [67, 72, 82, 102]. An expansion of sampling and molecular typing, particularly from a range of Australian marsupials and geographically distinct wombat populations, is clearly required for convincing phylogenetic inference.

2.6 Global attempts to study *S. scabiei* origin and spill-over

More broadly there have been three approaches either to understand spill-over or to infer origins of *S. scabiei* in the global literature: mite morphology, experimental cross-infections, and genetics. Minor morphological differences have classified *S. scabiei* into varieties (pathovars) [32, 67, 87, 108] with the presence or absence of dorsal and ventrolateral spines used as the primary differentiator [32]. These pathovars are simply named: *S. scabiei* var hominis, *S. scabiei* var *canis* and *S. scabiei* var *animal*, which can be distinguished further depending on the specific animal infested (e.g. *S. scabiei* var *wombatii*). Cross-infestations of

mites between different host species have also been shown to occur using this identification of pathovars, however, these documented spill-over events have typically been self-limiting [60-63, 89]. In terms of phylogenetic informativeness, a range of different gene loci have been used to attempt to answer questions about the relationship between mites isolated from different hosts. Outside Australia, genetic studies using a range of different genetic markers have revealed conflicting conclusions over whether geographic location and host has an impact on *S. scabiei* genetic structure [73, 101, 103, 109]. A detailed comparison of different gene loci can be seen in Table 2.1, with a total of 17 studies occurring during a 16 year period, spanning across 19 countries and 34 animal species. All studies were attempting to answer whether mites were genetically different depending on the host they were infecting and/or whether biogeographical separation existed. General conclusions from all gene targets include that (i) microsatellites identify distinctive host separation [71-73, 110-112], (ii) COX1 and 16S are consistent with host and location separation, with human specific mites indicating higher species separation based on location according to COX1 [67, 70, 72, 101, 107, 113], (iii) ITS-2 and 12S should only be used for *S. scabiei* identification and that a single species of mite infects all animals and humans [70, 82, 101-103, 107, 114, 115], and (iv) genes encoding for glutathione S-transferase-1 and voltage-sensitive sodium channels (GST1 and VSSC, respectively) might be a good indicator for host-related variation and resistance [116]. Interestingly, Erster et al. [116] found that COX1 did not play a role in mite host-specific separation in this particular study, which is contrast to other COX1 studies [101]. With genetic studies mentioned in Table 1.1 producing variable results, there is a clear need for increased consensus in the literature on the choice of genetic loci to address questions of *S. scabiei* spill-over and identify origins.

Table 2.1: Publicly available studies that have attempted to identify if *S. scabiei* can be genetically separated based upon location and/or host

Study	Host (Location)	Gene Target/ Conclusions
Zahler et al 1999 [114]	*Bos taurus* (Germany) *Camelus dromedarius* (Germany) *Canis lupus familiaris* (USA, India, Malaysia, New Zealand) *Lynx pardinus* (Sweden) *Nyctereutes procyonoides* (Japan) *Rupicapra rupicapra* (Austria) *Sus scrofa* (Germany, Belgium, Spain) *Vombatidae* (Australia) *Vulpes vulpes* (Sweden, Germany)	ITS-2: No separation due to location or host
Walton et al 1999 [73]	*Canis lupus familiaris* (Australia, USA) *Homo sapiens* (Australia, Panama) *Vombatus ursinus* (Australia)	Microsatellites: Human and dog derived mites cluster by host rather than location.
Skerrett et al 2002 [82]	*Canis lupus familiaris* (Australia) *Homo sapiens* (Australia) *Vombatus ursinus* (Australia)	12S rRNA: Wombats, dogs and humans had similar sequences
Berrilli et al 2002 [70]	*Rupicapra pyrenaica* (Spain) *Rupicapra rupicapra* (Italy) *Vulpes vulpes* (Italy, Spain)	ITS-2: No host or geographical separation 16S rRNA: Indicated significant differences between locations
Walton et al 2004 [72]	*Homo sapiens* (Australia, Panama) *Canis lupus familiaris* (Australia, USA) *Macropus* (Australia) *Pan troglodytes* (Tanzania)	16S: Produced three groups 1) human mites from Panama 2) Humans mites from Australia 3) mixed human and animal mites. COX1: Produced three groups 1) human mites from Panama 2)

	Vombatus ursinus (Australia) *Vulpes vulpes* (Sweden)	Humans mites from Australia 3) mixed human and animal mites. Microsatellites: Separated human mites into two distinct geographical clusters and further divide the animal mites into hosts groups.
Soglia et al 2007 [71]	*Capra ibex* (Italy) *Cervus elaphus* (Italy) *Martes foina* (Italy) *Martes martes* (Italy) *Ovis gmelini* (Italy) *Rupicapra pyrenaica* (Spain) *Rupicapra rupicapra* (Italy) *Sus scrofa* (France) *Vulpes vulpes* (Italy, Spain)	Microsatellites: Low levels of cross infections. Not strongly supportive of geographical separation within same host-specific varieties.
Gu and Yang 2008 [115]	*Oryctolagus* (China) *Sus scrofa* (China)	ITS-2: Single heterogeneous species
Alasaad et al 2009 [103]	*Capra ibex* (Italy) *Capra pyrenaica* (Spain) *Cervus elaphus* (Italy) *Martes foina* (Italy) *Ovis aries musimon* (Italy) *Rupicapra pyrenaica* (Spain) *Rupicapra rupicapra* (Italy) *Sus scrofa* (Italy, France) *Vulpes vulpes* (Italy, Spain, Switzerland)	ITS-2: Not suitable to identify genetic diversity among mites from different animals in different locations: monospecific

Rasero et al 2010 [110]	*Capra ibex* (Italy) *Capra pyrenaica* (Spain) *Cervus elaphus* (Italy) *Martes foina* (Italy, Spain) *Martes martes* (Italy) *Ovis aries musimon* (Italy) *Ovis aries musimon* (Italy) *Rupicapra pyrenaica* (Spain) *Rupicapra rupicapra* (Italy) *Sus scrofa* (Italy, France) *Vulpes vulpes* (Italy, Spain)	Microsatellites: Mites clustered into herbivore, carnivore and omnivore derived mite populations and the level of genetic exchange between mites from different locations is related to geographical distance.
Gakuya et al 2011 [112]	*Acinonyx jubatus* (Kenya) *Connochaetes taurinus* (Kenya) *Eudorcas thompsonii* (Kenya) *Panthera leo* (Kenya)	Microsatellites: Host-taxon specification with potentially predator/prey association.
Alasaad et al 2011 [61]	*Capreolus capreolus* (Spain) *Cervus elaphus* (Spain) *Rupicapra pyrenaica* (Spain) *Vulpes vulpes* (Spain)	Microsatellites: Herbivore, carnivore and omnivore separation.
Amer et al 2014 [101]	*Bos taurus* (Egypt) *Bubalus bubalis* (Egypt) *Oryctolagus* (Egypt) *Ovis aries* (Egypt)	ITS-2: No host segregation COX1: Host adaptation and geographically separated mites 16S: show host adaptation and geographically separated mites.
Zhao et al 2014 [107]	*Canis lupus familiaris* (China) *Homo sapiens* (China)	16S: Differentiate *S. hominis* from *S. animal* populations, but not as effective as COX1. COX1: Classified mites by different hosts with *S. hominis* further divided based on locations. ITS-2: No host or geographical preference

Makouloutou et al 2015 [113]	*Canis lupus familiaris* (Japan) *Capricornis crispus* (Japan) *Martes melampus* (Japan) *Meles anakuma* (Japan) *Nyctereutes procyonoides viverrinus* (Japan) *Procyon lotor* (Japan) *Sus scrofa leucomystax* (Japan)	ITS-2: Only good for identification of causative agent. 16S: showed minor genetic differences regardless of hosts in Japan. COX 1: Showed minor genetic differences regardless of hosts in Japan.
Erster et al 2015 [116]	*Canis aureus* (Israel) *Erinaceus concolor* (Israel) *Orictolagus cuniculus* (Israel) *Vulpes vulpes* (Israel)	COX1: Did not indicate host preference. GST1: Differences in host preference VCCS: Differences in host preference.
Andriantsoanirina et al 2015 [102]	*Homo sapiens* (France) *Canis lupus familiaris* (France)	12S rRNA: Using Skerratt et al 2002 haplotypes concluded that a single French human sequence was identical to the reference *S. scabiei* var *wombatii*.
Andriantsoanirina et al 2015 [67]	*Homo sapiens* (France)	COX1: Identified three genetically distinct clades: two clades exclusive to humans and one clade with a mix of both animals and humans. One of the two human clades had a mix of Australian and French samples.

2.7 Reanalysis of Australian studies

In addition to improvements of choice of genetic loci, analysis by contemporary analytical approaches can also contribute value to the emerging picture of mange origins in Australian wildlife. While it is beyond the scope of this review to add new genetic data, we apply a contemporary analytical approach to existing data. In order to represent the, often conflicting, phylogenetic signal in the available data, we turn to phylogenetic networks. In such networks, groups of taxa are split by sets of parallel lines, whose lengths correspond to the strength of phylogenetic signal splitting the taxa in that way, rather than simply by single branches of a tree [117]. By including bootstrap values greater than 80, the robustness of each network split can be analysed.

To understand how Australian mites cluster in the global mite population using a phylogenetic network, *S. scabiei* 16S rDNA and COX1 were obtained from GenBank for neighbour-net tree analysis using SplitsTree (version 4.13.1) [117]. Sequences from human and animal *S. scabiei* mites were obtained across Italy, Spain, China, Egypt, Australia, Panama, Japan, North America and Tanzania (Table 2.2). The outcomes from these two networks targeting these two genetic loci produce slightly different results. 16S rRNA gene sequence analysis showed two very distinct clades; sequences from human and animal European *S. scabiei* in one clade and sequences from human and animal *S. scabiei* from the rest of the world in the other clade (Fig. 2.1). Within the non-European clade there is limited support for further supplementary subclades. Interestingly, *S. scabiei* mites from Australian hosts are shown to be clustering very closely to Egypt, Japan and China derived mites. This may suggest that the once thought European origin of Australian *S. scabiei* could be incorrect, and perhaps the Australian *S. scabiei* associates more with Asian roots, which are clearly separate from the European derived mites. COX1 sequence analysis did not produce the same location separation as did the 16S rRNA gene analysis, but rather showed separation of human and animal sequences, except for

Table 2.2: 16S rRNA gene and COX1 sequences retrieved from GenBank. Each sequence is labelled as follows: Representative number associated to its corresponding Neighbour-net tree_Host_Location_Accession Number.

16S rRNA Sequences	COX1 Sequences
1_Rabbit_Egypt_AB779582	1_Human_Australia_AY493385
2_Rabbit_Egypt_AB779583	2_Human_Australia_AY493388
3_Rabbit_Egypt_AB779580	3_Human_Australia_AY493390
4_Rabbit_Egypt_AB779579	4_Buffalo_Egypt_AB779589
5_Rabbit_Egypt_AB779578	5_Buffalo_Egypt_AB779590
6_Rabbit_Egypt_AB779576	6_Buffalo_Egypt_AB779591
7_Rabbit_Egypt_AB779575	7_Buffalo_Egypt_AB779593
8_Rabbit_Egypt_AB779574	8_Buffalo_Egypt_AB779592
9_Rabbit_Egypt_AB779573	9_Buffalo_Egypt_AB779595
10_Rabbit_Egypt_AB779572	10_Dog_Australia_AY493394
11_Buffalo_Egypt_AB779564	11_Dog_USA_AY493393
12_Cattle_Egypt_AB779581	12_Wallaby_Australia_AY493398
13_Sheep_Egypt_AB779586	13_Wombat_Australia_AY493397
14_Sheep_Egypt_AB779585	14_Dog_China_KJ499544
15_Sheep_Egypt_AB779584	15_Dog_China_KJ748528
16_Sheep_Egypt_AB779587	16_Dog_China_KJ748529
17_Rabbit_Egypt_AB779577	17_Dog_China_KJ748527
18_Buffalo_Egypt_AB779570	18_Dog_Australia_AY493391
19_Buffalo_Egypt_AB779571	19_Dog_Australia_AY493392
20_Buffalo_Egypt_AB779565	20_Dog_USA_AY493395
21_Buffalo_Egypt_AB779566	21_Human_Australia_AY493382
22_Buffalo_Egypt_AB779567	22_Human_Australia_AY493383
23_Buffalo_Egypt_AB779568	23_Human_Australia_AY493384
24_Buffalo_Egypt_AB779569	24_Chimp_Tanzania_AY493396
25_Dog_China_KJ781369	25_Buffalo_Egypt_AB779594
26_Dog_China_KJ781371	26_Cattle_Egypt_AB779607
27_Dog_China_KJ781370	27_Buffalo_Egypt_AB779588
28_Dog_China_KJ781373	28_Rabbit_Egypt_AB779604
29_Human_China_KJ781376	29_Rabbit_Egypt_AB779603
30_Human_China_KJ781378	30_Rabbit_Egypt_AB779605
31_Human_China_KJ781374	31_Rabbit_Egypt_AB779598
32_Human_China_KJ781377	32_Rabbit_Egypt_AB779597
33_Human_China_KJ781375	33_Rabbit_Egypt_AB779599
34_Dog_China_KJ781372	34_Rabbit_China_EU256389
35_Dog_Japan_AB821002	35_Swine_China_EU256387
36_Raccoon_Dog_Japan_AB820995	36_Rabbit_China_EU256388
37_Raccoon_Dog_Japan_AB821003	37_Rabbit_China_EU256386
38_Raccoon_Dog_Japan_AB820998	38_Sheep_Egypt_AB779608
39_Raccoon_Dog_Japan_AB820997	39_Sheep_Egypt_AB779609
40_Raccoon_Dog_Japan_AB820999	40_Sheep_Egypt_AB779602
41_Raccoon_Dog_Japan_AB820996	41_Sheep_Egypt_AB779610

42_Japanese_Marten_Japan_AB821000
43_Japanese_Serow_Japan_AB821001
44_Dog_Australia_AY493410
45_Wallaby_Australia_AY493412
46_Chimp_Tanzania_AY493411
47_Human_Australia_AY493402
48_Dog_Australia_AY493409
49_Human_Australia_AY493403
50_Human_Australia_AY493404
51_Human_Australia_AY493408
52_Human_Australia_AY493405
53_Human_Australia_AY493406
54_Human_Australia_AY493407
55_Human_Panama_AY493401
56_Human_Panama_AY493399
57_Human_Panama_AY493400
58_Alpine_Chamois_Spain_AF311951
59_Red_Fox_23B_Italy_AF387691
60_Pyrenian_Chamois_Spain_AF387687
61_Red_Fox_23A_Italy_AF387695
62_Red_Fox_19D_Italy_AF387696
63_Alpine_Chamois_1A_Italy_AF387692
64_Alpine_Chamois_1B_Italy_AF387685
65_Alpine_Chamois_1C_Italy_AF387686
66_Red_Fox_19C_Italy_AF387697
67_Alpine_Chamois_18B_Italy_AF387698
68_Alpine_Chamois_5_Italy_AF387694
69_Alpine_Chamois_18A_Italy_AF387699
70_Alpine_Chamois_15H_Italy_AF387693
71_Alpine_Chamois_15G_Italy_AF387689
72_Alpine_Chamois_15F_Italy_AF387688
73_Red_Fox_20B_Spain_AF387700
74_Red_Fox_20C_Spain_AF387702
75_Red_Fox_20A_Spain_AF387701
76_Red_Fox_3A_Italy_AF387690
77_Red_Fox_4A_Italy_AF387680
78_Red_Fox_4B_Italy_AF387681
79_Red_Fox_16A_Italy_AF387676
80_Red_Fox_4C_Italy_AF387683
81_Red_Fox_6A_Italy_AF387679
82_Red_Fox_6B_Italy_AF387682
83_Red_Fox_8A_Italy_AF387675
84_Red_Fox_8C_Italy_AF387678
85_Red_Fox_8B_Italy_AF387677
86_Red_Fox_21_Italy_AF387684
87_Human_Italy_AF495527

42_Sheep_Egypt_AB779611
43_Rabbit_Egypt_AB779601
44_Rabbit_Egypt_AB779606
45_Rabbit_Egypt_AB779600
46_Rabbit_Egypt_AB779596
47_Human_China_KJ748524
48_Human_China_KJ748523
49_Human_China_KJ748525
50_Human_China_KJ748521
51_Human_China_KJ748522
52_Human_China_KJ748526
53_Human_Panama_AY493379
54_Human_Panama_AY493380
55_Human_Panama_AY493381

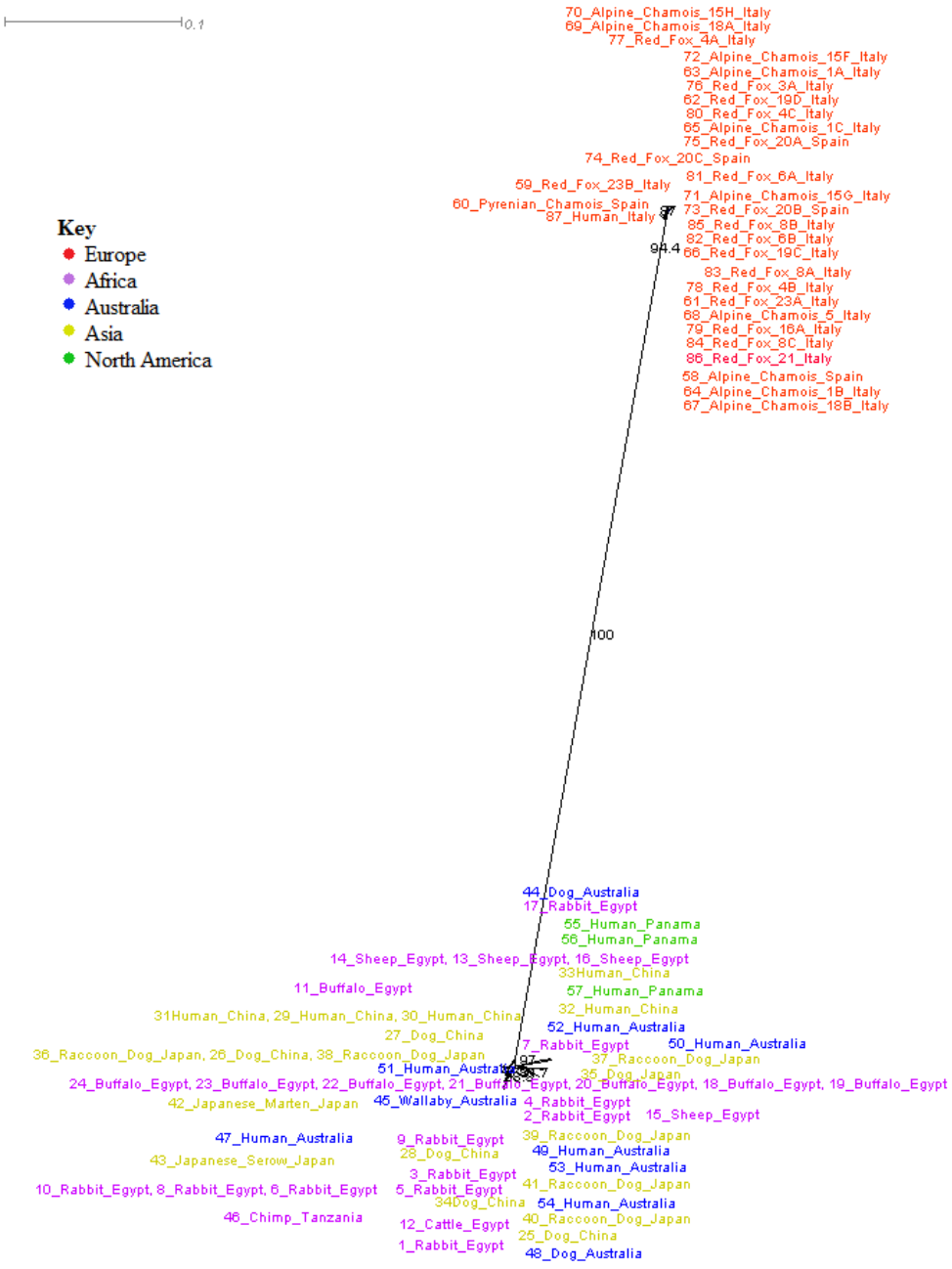

Figure 2.1: Neighbour-net analysis using SplitsTree of publicly available 16S rRNA gene sequences retrieved from GenBank (July 2015). Bootstrap values greater than 80 are included. Human and animal *S. scabiei* mites from Europe cluster away from other global *S. scabiei* mites. The Australian derived mites are shown to cluster closely with Asian and African mites, which conflicts with the assumption that Australianmites are consequential to European origins. There is limited network support for internal subclades in both the European clade and the Asian, Australian, African and North American clade

three human *S. scabiei* mites which were found in the animal clade (Fig. 2.2). Due to the lack of European derived mites for COX1, it is hard to accurately conclude whether European origins have an influence on this clade separation. Comparing the animal host clade of COX1 sequence analysis to the non-European clade of 16S rRNA gene sequence analysis, COX1 produced higher internal clade support for sequence separation than 16S rDNA. This suggests that COX1 may be more selective for higher divergence of *S. scabiei* than 16S rRNA gene.

Both neighbour-nets do produce an overall interpretation that geographic location and host species play a distinct role in mite separation. This supports that *S. scabiei* is frequently host-specific with periodic host spill-over events. An interesting feature to note is that one study that used human *S. scabiei* mites from China (sample numbers 47–52) analysed only 45 % (approximately 317 bp) of the COX1 sequence compared to the longer sequence (approximately 1,448 bp) analysed by others (sample numbers 15–17 and 34–37). The remaining sequences were all roughly around 747 bp. Trimming all available COX1 sequences to 314 bp for SplitsTree analysis did not produce any significant changes to the overall outcome. However, the wallaby and wombat *S. scabiei* sequences were not separated in the neighbour-net as unique single sequences, but rather clustered on a node within the network. This emphasises the additional value of sequencing larger gene sequence fragments where possible, since phylogenetically informative areas of the gene may be excluded when limiting sequence length, which in turn may strongly influence the outcomes of such analysis.

By adding additional new *S. scabiei* samples and solving some of the genetic loci problems, as discussed, greater consensus may be reached as to the origin of mange in Australian wildlife. We propose that there are several alternative hypotheses that may be revealed about the mechanism of spill-over, its frequency, and timescale from improved *S. scabiei* phylogenetics: (i) mange was already present within Australian wildlife such as dingos before the arrival of European settlers; (ii) there was a single introduction event from original European settlement;

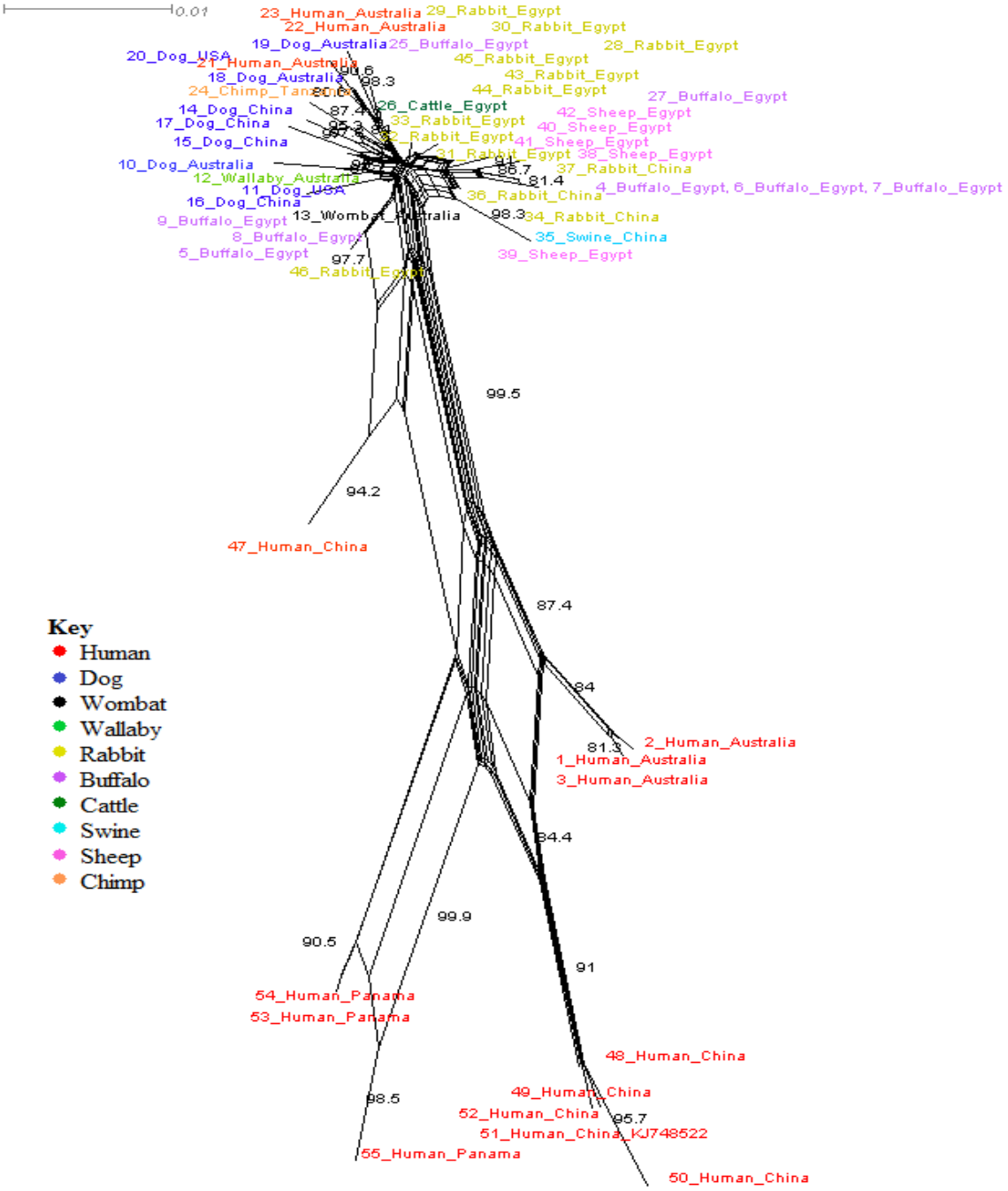

Figure 2.2: Neighbour-net analysis using SplitsTree of publicly available COX1 sequences retrieved from GenBank. Bootstrap values greater than 80 are included. COX1 sequences analysis supports host-separation rather than geographic location is the biggest influence on *S. scabiei* diversity. Dog, wallaby and wombat sequences are shown to be clustering closely. The majority of sequences are branching away independently within both clades as unique sequences.

(iii) there were multiple events of introduction since European settlement from other ethnic regions; and (iv) combinations of these hypotheses (Fig. 2.3, illustrating the combination of all three). To resolve these hypotheses, supplementary sampling needs to occur, including increased sampling from dingos, other canids and wombats to answer questions over the genetic diversity of *S. scabiei* mites within Australian wildlife. Additionally, sampling from humans, canids and other animals that are prone to high morbidity of *S. scabiei* in endemic countries would help resolve the genetic timeline globally.

Alternatively, the advent of whole genome sequencing of nuclear or mitochondrial DNA may replace these gene-specific analyses altogether [118, 119]. In the absence of whole genome sequencing, we recommend using COX1 gene molecular typing for *S. scabiei* host species specific separation. Additionally, perhaps rather than simply asking "are the mites different?" more explicit hypothesises about likely mechanisms, timescales and frequency of spill-over or origins should be addressed. For example, it is notable that while the past studies have either agreed or disagreed on host and/or location to be a definitive key to different *S. scabiei* mites, the use of sequence data to infer divergence times using ancestral state reconstructions has never been addressed [120].

2.8 Conclusions and future directions

New pathogens in a novel organism have the potential to cause high morbidity and mortality to animals that have not previously been exposed or have evolved defences [121]. Such knowledge can also be critical for justifying disease management, owing to perceived invasiveness. The settlement of Europeans and their livestock into Australia since 1788 has introduced new pathogens, with sarcoptic mange in Australian wildlife proposed to be one of several important examples [59]. This scenario is unlikely to be unique in Australia with growing molecular evidence that the obligate intracellular bacteria, *Chlamydia pecorum*, and

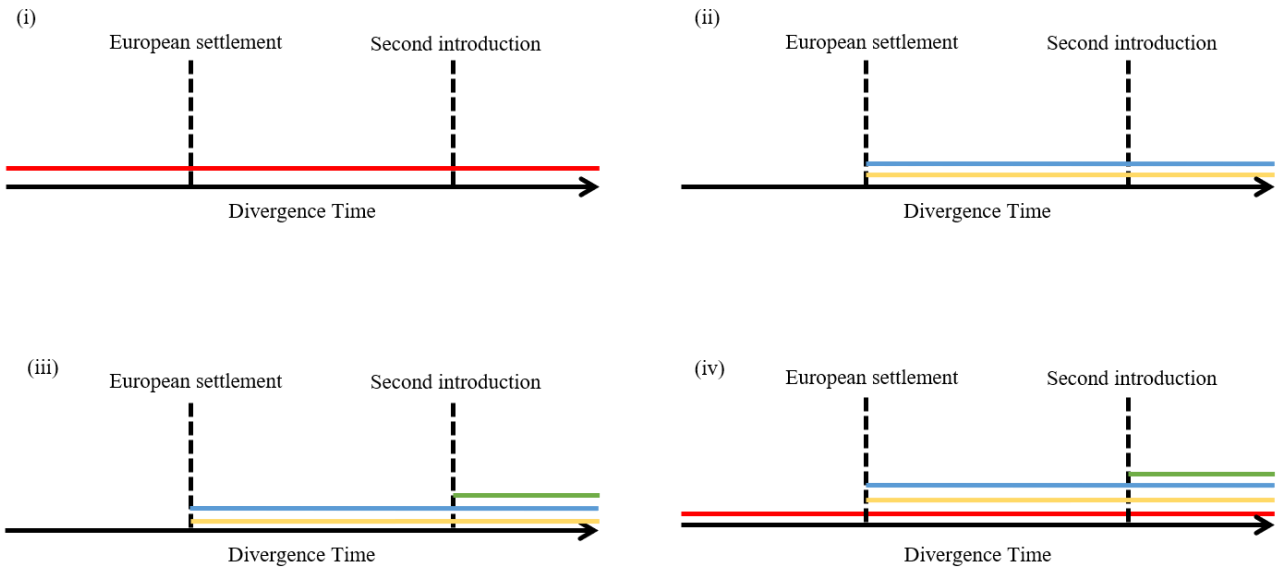

Figure 2.3: Representation of four different scenarios of how mites were introduced into Australian wildlife. Each line represents a different host, introduction period and are genetically unique. (i) *S. scabiei* was already present in Australian wildlife via the dingo prior to European settlement (ii) a single manifestation from European settlers and their domestic dogs (iii) after initial European settlement, a second and new introduction of *S. scabiei* was introduced from other regions across the world and (iv) combinations of all three situations (illustrating all three at once). Colours indicate species as follows: red - dingo, yellow - European domestic dogs, blue - European settlers, and green - second introduction of mites from other ethnic countries. Each of these possible scenarios would produce different clade structures on a phylogenetic tree, respectively as follows: (i) a single Australian *S. scabiei* subclade within the larger *S. scabiei* phylogeny with divergence time pre-dating European arrival, (ii) single or two Australian *S. scabiei* subclades within the larger phylogeny with divergence time associated to European arrival, (iii) a further subclade within (ii) associated to reintroduction times, and (iv) a single *S. scabiei* subclade within the larger *S. scabiei* phylogeny distinctly separate from the smaller subclades associated to European settlement and other more recent introductions.

major pathogen of the iconic Australian marsupial, the koala, may have origins in spill-over from introduced livestock carrying this pathogen [122, 123].

To date, the most convincing conclusion is that sarcoptic mange in Australia was introduced by settlers and their dogs, and subsequently became a major disease burden to native wildlife. This review has discussed the conflicting results of phylogenetic studies of sarcoptic mange and highlighted the need to establish a more consistent and robust set of genomic loci for analysis. We conclude that of all available gene loci that have been used, a combination of both genomic (e.g. microsatellites) and mitochondrial (COX1) loci should be combined for host and location separation to have the best chance to eliminate phylogenetic conflict. Genes encoding for GST1 and VSSC may be equally important as these genes are related to immune resistance; however, further research is needed to confirm this, and to expand available sequences for comparison. In light of this reanalysis, perhaps an additional question to explore is "since genetic differences exist between mites infecting different hosts and locations, do these differences occur in key genes that can influence disease states and pathogenicity, and is there a more selective gene that can better identify mite variation?". This can also be simplified as simply that better markers are needed, along with the right samples to assess them.

Mange in Australian wildlife illustrates the importance of sarcoptic mange due to its continual increase in host range and global diversification [5]. Future genetic and phylogenetic research will contribute valuable knowledge applicable to wildlife conservation and the health to both humans and animals infected with *S. scabiei* (a Conservation Medicine and One Health framework).

CHAPTER 3

MITOCHONDRIAL GENOME SEQUENCING REVEALS POTENTIAL ORIGINS OF THE SCABIES MITE *SARCOPTES SCABIEI* INFESTING TWO ICONIC AUSTRALIAN MARSUPIALS

Tamieka A. Fraser[1,2], Renfu Shao[2], Nicholas M. Fountain-Jones[3], Michael Charleston[1,4], Alynn Martin[1], Pam Whiteley[5], Roz Holme[6], Scott Carver[1] and Adam Polkinghorne[2*]

[1] School of Biological Sciences, University of Tasmania, Sandy Bay, Hobart, TAS, Australia.

[2] Centre for Animal Health Innovation, School of Science and Engineering, University of the Sunshine Coast, Sippy Downs, QLD, Australia.

[3] University of Minnesota, Minneapolis, MN, USA.

[4] School of Information Technologies, University of Sydney, Camperdown, NSW, Australia.

[5] Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Werribee, VIC, Australia.

[6] Cedar Creek Wombat Rescue Inc. & Hospital, Cedar Creek, NSW, Australia.

This paper was published in Parasites & Vectors, 2017. 17(1): p. 233

Statement of joint authorship

Conceived and designed experiments: TF, AP, SC, and RS. Collected samples: PM, AM, RH and TF. Phylogenetic analysis: TF, NFJ, RS and MC. Wrote the paper: TF, NFJ, RS, MC, RH, AM, PM, SC and AP. All authors read and approved the final manuscript.

3.1 Abstract

Background: Debilitating skin infestations caused by the mite, *Sarcoptes scabiei*, have a profound impact on human and animal health globally. In Australia, this impact is evident across different segments of Australian society, with a growing recognition that it can contribute to rapid declines of native Australian marsupials. Cross-host transmission has been suggested to play a significant role in the epidemiology and origin of mite infestations in different species but a chronic lack of genetic resources has made further inferences difficult. To investigate the origins and molecular epidemiology of *S. scabiei* in Australian wildlife, we sequenced the mitochondrial genomes of *S. scabiei* from diseased wombats (*Vombatus ursinus*) and koalas (*Phascolarctos cinereus*) spanning New South Wales, Victoria and Tasmania, and compared them with the recently sequenced mitochondrial genome sequences of *S. scabiei* from humans.

Results: We found unique *S. scabiei* haplotypes among individual wombat and koala hosts with high sequence similarity (99.1% - 100%). Phylogenetic analysis of near full-length mitochondrial genomes revealed three clades of *S. scabiei* (one human and two marsupial), with no apparent geographic or host species pattern, suggestive of multiple introductions. The availability of additional mitochondrial gene sequences also enabled a re-evaluation of a range of putative molecular markers of *S. scabiei*, revealing that cox1 is the most informative gene for molecular epidemiological investigations. Utilising this gene target, we provide additional evidence to support cross-host transmission between different animal hosts.

Conclusions: Our results suggest a history of parasite invasion through colonisation of Australia from hosts across the globe and the potential for cross-host transmission being a common feature of the epidemiology of this neglected pathogen. If this is the case, comparable patterns may exist elsewhere in the 'New World'. This work provides a basis for expanded molecular studies into mange epidemiology in humans and animals in Australia and other geographic regions.

Keywords: *Sarcoptes scabiei*, Wombat, Koala, Mitochondrial genome sequencing, cox1, Phylogeny, Conservation

3.2 Background

Estimated as infecting 110 million humans and more than 100 mammalian species worldwide, *Sarcoptes scabiei* is widespread, burdensome, and has among the widest host range of known parasites [20, 124]. *S. scabiei* infests the epidermis of its hosts, causing a wide range of host immune responses and associated mange disease symptoms (otherwise classified as scabies in humans) including skin irritation, inflammation, hyperkeratosis, alopecia, pruritis, rheumatic heart disease, and secondary bacterial infections [20]. The life cycle of this mite has five developmental stages (egg, larva, protonymph, tritonymph and adult) occurring within the stratum corneum, with durations of life cycle occurring between 7 and 21 days [14, 21, 125]. It is most commonly reported in humans, domestic dogs and livestock, with impacts to health, animal welfare and primary production [5, 23, 125]. Transmission of mites between individuals and hosts occurs by direct contact, sharing of contaminated materials or habitat with mites known to survive off its host for up to three weeks [14, 21]. With a wide and expanding wildlife host range, this pathogen is also labelled an emerging infectious disease in North America and Australia [5].

Sarcoptic mange or scabies is a major parasitic disease of indigenous people and their domestic dogs, particularly in northern Australia [72, 126, 127]. In remote Aboriginal communities, up to 50% of children suffer from *S. scabiei* infestations, resulting in endemic transmission and severe cases of scabies induced pyoderma [127]. These communities also share communal space with mange infested dogs, a risk for continuous zoonotic transmission [72]. *S. scabiei* has also been documented to infest iconic Australian native species, including wombats (*Vombatus ursinus*, *Lasiorhinus latifrons*) [59], wallabies (*Wallabie bicolor*, *Macropus agilis*) [75, 76], koala (*Phascolarctos cinereus*) [92], southern brown bandicoot (*Isoodon obesulus*) [77], and dingo (*Canis lupus dingo*) [23]. Among these, wombats appear to experience the most severe pathology with mange associated with population declines exceeding 90% in some instances [40, 58]. Recent studies suggests a rise in mange cases in koalas also, particularly in populations from southern Australia [74].

Despite large numbers of mange cases in these wildlife species, the origins and phylogenetic relationships of *S. scabiei* among host species remains significantly understudied. Only two studies have contributed to the genetic knowledge of Australian-derived mites and these have revealed contrasting results. The first of these studies utilized sequencing of partial *S. scabiei* 12S rRNA gene sequence fragments [82], revealing close sequence similarity between mites from wombats, dogs and humans in Australia [82]. A subsequent investigation examining mites from Australian humans and animals and comparing them to mites from elsewhere, however, highlighted a strong relationship between *S. scabiei* mitochondrial DNA haplotypes based on host and geographic differences [72]. While no new sequences were obtained from Australian animals, most recently, a French study of 12S rRNA and *cox1* gene sequences from *S. scabiei* isolated from humans and dogs supported Skerratt et al.'s [82] original suggestion of a relationship between human, domestic animal and wombat sequences. It is, thus, proposed that occurrence of sarcoptic mange in wombats is the result of past spill-over from humans and

dogs, potentially from Europe. The exact relationship between mites infesting native Australian wildlife and the endemic strains shared between indigenous human populations and domesticated dogs is currently unknown due to the almost complete absence of any additional sequence data from these sources of *S. scabiei* [128].

Apart from the chronic shortage of genetic information for *S. scabiei*, considerable controversy exists over the choice of gene loci to be used for molecular typing of this pathogenic mite. The most common targets for *S. scabiei* typing studies to date have been the mitochondrial 12S rRNA, 16S rRNA and Cytochrome oxidase 1 (*cox1*) genes. The 12S rRNA gene is highly conserved and does not discriminate well between host species infected by *S. scabiei*, although some studies have used it for this purpose [82, 102]. The 16S rRNA gene is less conserved, though is also limited in its ability to identify genetic distinctions among *S. scabiei* mites separate hosts or locations [113, 129]. *cox1* is the most variable of the three genes, with greater power to distinguish between mites from different host species [101, 107, 128, 130]. Nuclear markers have also been investigated including the use of microsatellites [71, 110-112] and phylogenetic analysis of ITS-2 gene sequences [70, 101, 103, 113-115]. The latter studies concluded that *S. scabiei* was a single heterogeneous species with a low level of genetic diversity [70, 101, 103, 113-115]. Microsatellites studies have provided some genetic evidence for cross-species infestation [110], with some limited ability to distinguish mites from different host groups but not by geographic location of isolation [71, 110-112].

In an effort to gain insight into the origins of *S. scabiei* in Australian wildlife and to provide base-line data for studies to investigate the relationship to *S. scabiei* infestations of humans and domesticated animals, we sequenced the mitochondrial genomes of *S. scabiei* mites collected from bare-nosed wombats from Tasmania, New South Wales (NSW) and Victoria (VIC), and koalas from VIC. We used this new genetic information to assess the potential role of *S. scabiei* cross-host transmission to and from marsupial host species following detailed phylogenetic

analysis. Because the choice of genetic loci for molecular epidemiology studies has influenced previous studies of *S. scabiei* origins and host specificity, we additionally examined individual mitochondrial genes to aid in the selection of gene targets for downstream investigations into the phylogenetic relationships of mites from a range of hosts and geographic locations, not only for Australian studies but for global comparisons.

3.3 Methods

3.3.1 Mite collection and DNA extraction

Mites were obtained via skin scrapings during necropsy from Victorian koalas (N= 5) and wombats from Victoria (N= 2) and Tasmania (N= 3) or as a part of routine veterinary care from anaesthetised New South Wales wombats (N = 2). From each individual host, 1–3 mites were pooled for DNA extraction (to obtain sufficient mtDNA for sequencing) following protocol using a DNeasy Blood and Tissue kit (Qiagen). The term "mite" will be used here forth for pooled sequences of several individuals.

3.3.2 Mitochondrial DNA long range PCR

To amplify full-length mitochondrial DNA sequences, primers were designed to cover four overlapping fragments (Table 3.1). Each long-range PCR was conducted on a thermocycler with a final PCR volume of 20 µL; 5 µL5X PrimeSTAR® GXL DNA buffer (Takara), 2 µL dNTP mixture (2.5 mM), 1 µL PrimeSTAR® GXL DNA Polymerase (Takara), 1 µL of forward and reverse primers (0.3 µM), 9 µL water and1 µL of DNA template. Cycling conditions consisted of 35 repeats of 98 °C for 10 s, 50 °C for 20 s and 68 °C for 10 mins. The presence of amplicons of the expected size was visually verified on a 1% TBE agarose gel, prior to purification using QIAquick PCR purification kit (Qiagen). DNA sequences of purified amplicons were determined by paired-end sequencing on a HiSeq4000 (BGI, Hong Kong) at 100 Mb coverage after passing quality control.

Table 3.1: Primer details for long range PCR. The four long range PCR fragments spaning across the mitochondrial genome are 4.4 kb, 4.0 kb, 3.8 kb and 1.7 kb long. These primers are positioned on four main genes; 12S rRNA gene, *nd4*, *cob* and *cox1*.

Fragment Length	Gene direction	Primer 1 (5' – 3')	Gene direction	Primer 2 (5' – 3')
4.4kb	12S rRNA forward	CAAGTTCCTGTGAATATATAGAAAACCGCC	*cox1* forward	GGACACCCGGAAGTTTACATTC
4.0kb	*nd4* forward	CCAAAAGCTCATGTAGAAGCTCCTCTAGAAGG	*cob* reverse	TAGTTACTCCTACTCATATTCAACC
3.8kb	*nd4* reverse	CCTTCTAGAGGAGCTTCTACATGAGCTTTTGG	12S rRNA reverse	GGCGGTTTTCTATATATTCACAGGAACTTG
1.7kb	*cob* forward	GGAACTGAACGAAGAATAGCATAAGC	*cox1* reverse	GAATGTAAACTTCCGGGTGTCC

3.3.3 *S. scabiei* mitochondrial genome assembly and annotation

Read mapping was performed in Geneious 9.1.3 [131] using the Geneious mapper method, originally using the four primer pair sequences used for PCR amplification as the reference sequence at 5 iterations, 100% identity and a minimum overlap of the same length of the primers (*cox1* at 22 bp, 12S rRNA at 30 bp, cob at 26 bp and nd4 at 32 bp). Reads were then mapped to the four output contigs at 100 bp overlap, 99% identity and 100 iterations. Contigs produced from this assembly were aligned using MUSCLE alignment [132] in Geneious version 9.1.3 [131]. Assembly of the new mitochondrial genome sequences obtained in this study was confirmed by sequence alignment against the previously published human *S. scabiei* mitochondrial genome (accession number LN874268).

Mitochondrial protein coding genes were annotated by identifying open reading frames (ORF) in Geneious and blastp to confirm ORF identification and protein length. tRNA genes were identified using ARWEN [133]. The two rRNA genes were identified by blastn search of GenBank [134]. DnaSP [135] was used to assess the magnitude selective pressure by dN/dS rations for all protein coding genes.

3.3.4 Phylogenetic tree analysis

The relationship between the new *S. scabiei* mitochondrial genomes and the available human derived mite was conducted by neighbour-net analysis at 1000 bootstrap replicates using SplitsTree (version 4.14.4) [117], showing only bootstrap values greater than 80. Individual full length gene tree outputs of 16S rRNA, *cox1*, *cox3*, *cytb* and ND4 were constructed with Mr. Bayes [136] in Geneious version 9.1.3, and were then compared to the Mr. Bayes tree output produced by the near full length (NFL) mitochondrial genome. Node tips and clade outputs were then visually compared between the two trees of interest, with associated tip lines drawn between the trees for clarity.

Global *cox1* phylogenetic analysis was completed using the current 12 mitochondrial genomes and the 78 available *cox1* sequences in GenBank. All *cox1* sequences were unified by trimming to 387 bp to allow alignment of sequences of the same length. Phylogenetic tree construction on the resulting 90 sequences was performed by Mr. Bayes and rooted with *Otodectes cynotis* mitochondrial genome (KP676688). Bootstrap values greater than 80 were shown.

3.4 Results

3.4.1 *S. scabiei* mitochondrial genome assembly and annotation identify new haplotypes in Australian marsupials

Successful full-length mitochondrial genome assembly was confirmed for all samples (accession numbers MF083732-MF083743). Neither single nor pooled DNA extraction played a significant role in amplification or exhibited sequencing errors. The mitochondrial genome size ranged between 13,830 bp and 13,926 bp with variations in length largely owing to the non-coding repeat region of "ATs". The mitochondrial genome GC content was comparable amongst all samples, and all new *S. scabiei* from koala and wombats were >98% similar to the mite genome from humans (Table 3.2).

Thirteen protein coding genes were identified in all 12 mitochondrial genomes using the invertebrate mitochondrial genetic code. tRNA annotation (ARWEN) resolved the presence of 16 of the 22 tRNA genes, with a further four added (trnQ, trnR, trnP and trnF) based on a visual inspection of the mitochondrial genome sequences and sequence identity to the previously annotated human *S. scabiei* mitochondrial genome (LN874268) [119]. Two invertebrate tRNA genes (trnA and trnY) were not identified, however these were also missing in the human *S. scabiei* mitochondrial genome (LN874268) [119].

Sequence analysis of the wombat and koala mite mitochondrial genomes, after removing the repeat region variation between trnS1 and trnF, revealed the presence of 11 unique haplotypes

Table 3.2: Details of new mitochondrial genomes, size, GC content and their sequence similarity to the human *S. scabiei* mite (LN874268).

Sample Name	Host (Location)	Pooled/Single Mite	Genome Size (bp)	GC %	% Similarity to LN874268
DW02	Wombat (Narawntapu, Tasmania)	Single	13911	19.21	99.44
S1	Wombat (South Swansea, Tasmania)	Single	13839	19.37	99.03
B1	Wombat (Brighton, Tasmania)	Single	13887	19.23	99.47
N11	Wombat (Central Coast, New South Wales)	Pooled	13845	19.39	98.62
N22	Wombat (Murrys Run, New South Wales)	Single	13900	19.32	98.98
W2	Wombat (Kangaroo Ground, Victoria)	Pooled	13856	19.33	99.06
VW	Wombat (Arthurs Creek, Victoria)	Pooled	13848	19.35	99.16
K2	Koala (Koonoomoo, Victoria)	Pooled	13926	19.29	99.43
K4	Koala (Koonoomoo, Victoria)	Pooled	13828	19.39	99.18
K5	Koala (Sandy Point, Victoria)	Pooled	13830	19.40	99.21
K7	Koala (Koonoomoo, Victoria)	Pooled	13854	19.35	98.86
K8	Koala (Koonoomoo, Victoria)	Pooled	13846	19.37	99.39

that shared between 99.1% and 100% sequence identity against the human *S. scabiei* mitochondrial genome. All NFL mitochondrial genomes were the same length when aligned (13,822 bp). Between the new genomes, only two koala mites (K4 and K7) shared 100% sequence similarity. The highest number of differences occurred between two wombat mites, N11 and DW02, from NSW and Tasmania respectively (171 nucleotides). The NFL mitochondrial genome of the human *S. scabiei* mite had the highest number of differences to a NSW wombat mite (N11) (178 nucleotide) and the lowest number of differences to a Victorian koala (K8) (83 nucleotides).

3.4.2 Phylogenetic analysis reveals a range of *S. scabiei* haplotypes among koalas and wombats

SplitsTree phylogenetic analysis of complete *S. scabiei* near full-length mitochondrial genomes revealed three major clades (bootstrap support >0.8), (Fig. 3.1); one is exclusively comprised of the human mite (LN874268) and the remaining two are a mix of marsupial mites. Each marsupial lineage could be further subdivided into two smaller clades comprising: (i) wombat mites from Tasmania (DW02 and Brighton), (ii) wombat (WV) and koala mites (K8 and K2) from Victoria, (iii) wombat mites from New South Wales (N11 and N22) and Victoria (W2), and; (iv) mites from a single wombat from Tasmania (S1) and koalas from Victoria (K4, K5 and K7). With the exception of subclade iii, all subclades are strongly supported by the length of parallel splits and their corresponding bootstraps. No clear host or geographical pattern was evident between the two major marsupial mite clades. We conducted a range of phylogenetic analyses all emitting the same result.

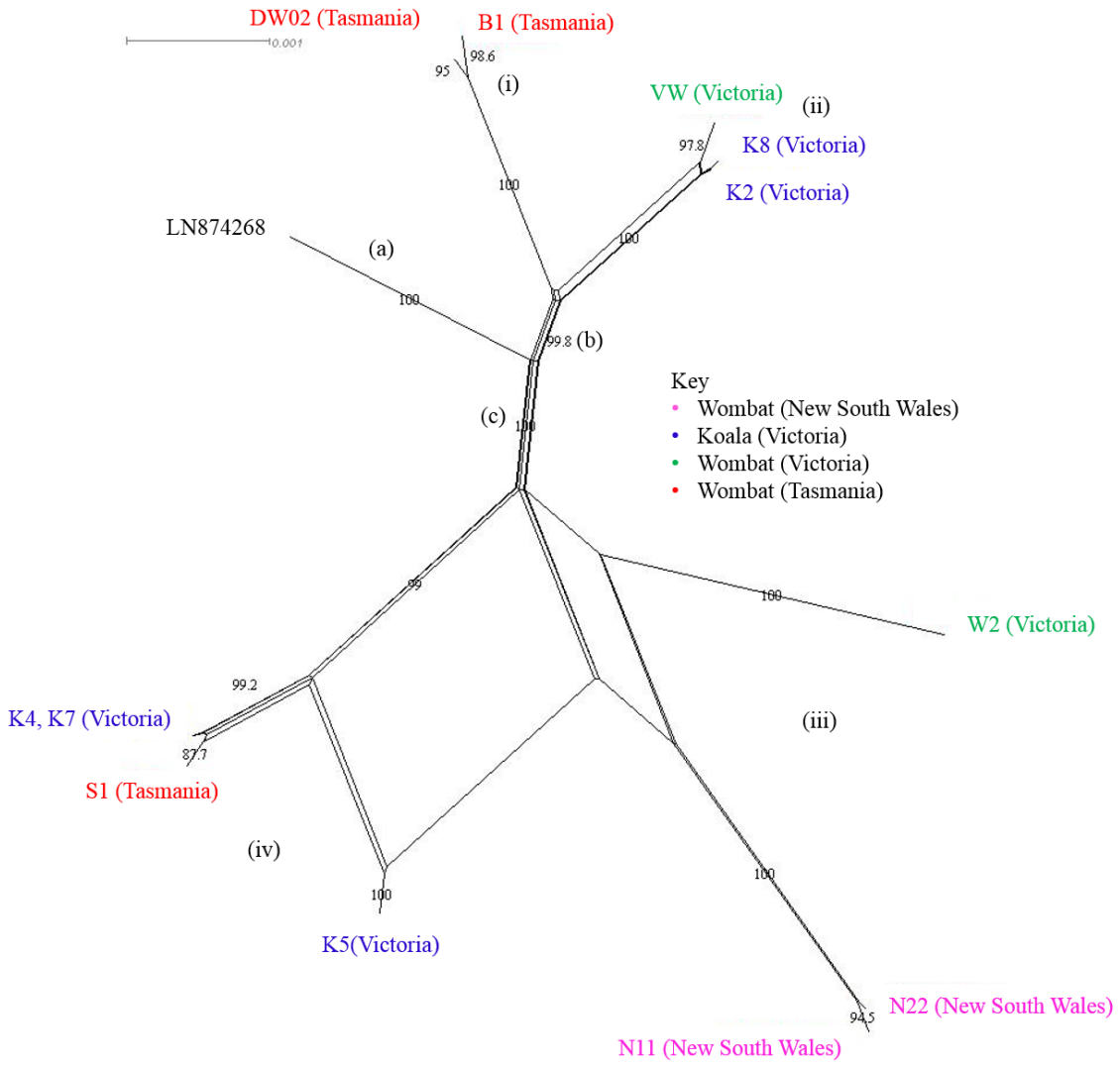

Figure 3.1: SplitsTree analysis of new near full-length mitochondrial genomes for koala and wombat *S. scabiei*. Three major clades are identified by splits: a) the human mite LN874269, b) marsupial mites from Tasmania and Victoria and, c) marsupial mites from New South Wales, Victoria and Tasmania. Four subclades are further recognized, labelled (i-iv).

3.4.3 Co-phylogenetic comparisons show individual mitochondrial genes vary in their utility for molecular epidemiological investigations

The expanded set of mitochondrial genome sequences for *S. scabiei* generated in this study enabled an evaluation of several mitochondrial genetic markers for molecular epidemiology studies. DnaSP [135] analysis revealed that (i) every mitochondrial gene is under negative selection with atp8 to be the closest to neutral selection, (ii) nd4 had the highest number of parsimony informative sites and, (ii) cytb identified the largest number of different haplotypes (Table 3.3).

Using the five genes with the greatest number of unique haplotypes (16S rRNA 8, *cox1* 9, cox3 8, cytb 10 and nd4 8), a co-phylogenetic comparison of their trees was made to the NFL phylogeny (Fig. 3. 2). All trees were rooted with the human mite (LN874269) to allow comparisons of clade construction and analysis of how the new haplotypes relate to each other. The phylogenetic trees constructed from each of the five genes portrayed the three major clades shown by analysis of the NFL mitochondrial genomes. Noticeably, the phylogenetic trees constructed on two of these genes (*cox1* and nd4) also revealed the same subclade structure. For cox3 and 16S rRNA genes, the low number of haplotypes identified revealed less resolution of putative ancestral linages. Cytb and 16 s rRNA genes differed with the position of K5 and W2 alternating between with the two NSW wombat mite clades and the VIC koala and Tasmanian wombat mite clade. The change in relative position of K5 and W2 in the latter trees expands a subclade that is predicted to include only wombat sequences based on NFL mitochondrial sequence analysis to also include sequences from koalas.

Table 3.3: Comparison of *S. scabiei* mitochondrial genes from koala and wombat hosts showing the number of informative sites, haplotypes and the magnitude of natural selection (dN/dS).

Gene locus	Length (bp)	Number of parsimony informative sites	dN/dS	Number of haplotypes
12S rRNA	657	4	NA	7
16S rRNA	1040	13	NA	8
atp8	159	4	0.705	4
cox1	1551	17	0.040	9
cox2	751	9	0.031	6
cox3	783	13	0.022	8
Cytb	1101	16	0.066	10
nd1	900	11	0.055	6
nd2	928	12	0.245	6
nd3	354	3	0.105	7
nd4l	255	3	0.000	3
nd4	1297	21	0.132	8
nd5	1626	20	0.040	7
nd6	441	4	0.000	5

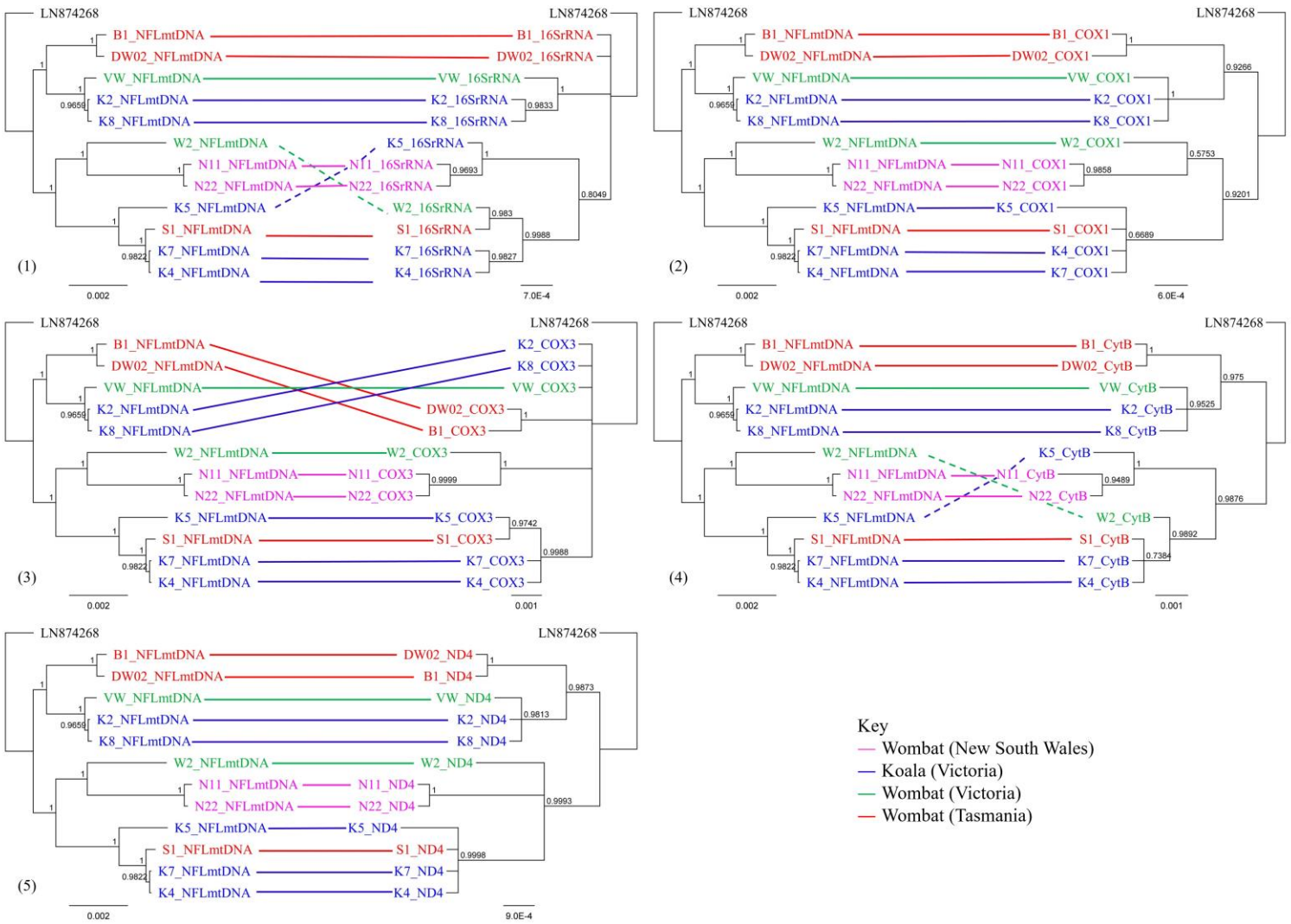

Figure 3.2: Comparison of tree output of NFL mitochondrial genomes to full-length individual gene trees. Comparison to NFL genomes include: 1 16S rRNA gene, 2 cox1, 3 *cox3*, 4 *cytB* and, 5 *nd4*. All trees were similar but only cox1 and nd4 produced identical clustering.

3.4.4 *cox1* analysis reveals that Australian marsupials share *S. scabiei* haplotypes with a diverse range of globally distributed mites

We combined our new *cox1* sequences with other global *cox1* haplotypes from across the globe [67, 128, 130] to perform a phylogenetic analysis to elucidate the relationships between Australian mites and those from animals and humans elsewhere. Sequence comparison and clade outgroups identified five haplotypes: (i) K7, S1, K4 and K5 were identical to a dog mite and three racoon dog mites from Japan (AB821011, AB821004, A821007, AB821012), (ii) W2 was identical to a dog mite from China (KT961022), a dog mite from Italy (KT961025), a single dog mite and three fox mites from France (KT961029, KT961030, KT961031, KT961032), a dog mite from the USA (AY493393) and a wombat mite and wallaby mite from Australia (AY493397 and AY493398), (iii) B1 and DW02 were the same and unique to everything else, (iv) VW, K2 and K8 are identical to a single dog mite from Australia (AY493391) and, (v) N11 and N22 were identical to a jackal mite, a buffalo mite and two sheep mites from Egypt (KP987792, AB779594, AB779608, AB779609). From the phylogenetic tree (Fig. 3.3) haplotypes containing the Tasmanian wombats (B1 and DW02) and Vic marsupials (VW, K2 and K8) are the only sequences that sit within other human sequences from Australia and France and dog sequences from China. All other human sequences are within its own distinct major clade.

3.5 Discussion

Pathogen dispersal and spill-over likely underscore global infestations of *S. scabiei* among many animal species, including Australian wildlife [87]. However, a dearth of genetic information for this mite has hindered molecular epidemiological investigations. We tackled this problem with mitochondrial genome sequencing of *S. scabiei* across a broad geographic range of two impacted marsupial host species. The comparison of NFL genome assemblies

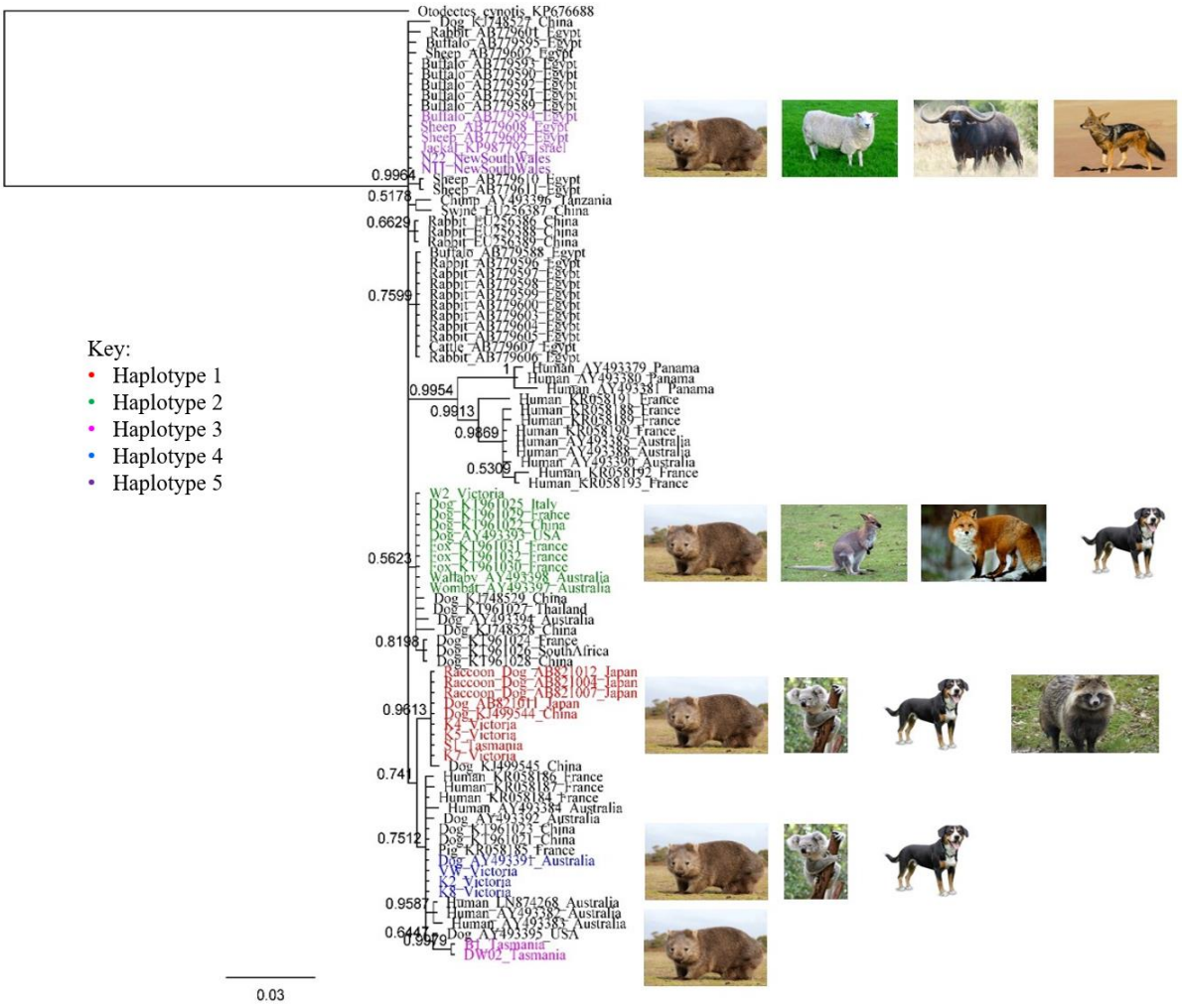

Figure 3.3: Phylogenetic analysis of global cox1 sequences. Comparison of 78 available cox1 sequences in GenBank were used to construct a global phylogenetic tree, including the current Australian mitochondrial genomes, with *Otodectes cynotis* mitochondrial genome (KP676688) as the outgroup. Sequence comparison and clade outgroups identified five haplotypes which the new marsupial mite sequences were identical to as shown by colour and images of hosts for each haplotype. Each sequence from GenBank is labels as Host_Accession Number_Location.

showed unique haplotypes infesting koalas and wombats across south-eastern Australia. Despite high NFL sequence similarity (>99%), our analysis revealed 11 unique mitochondrial haplotypes, with only two koala mites sharing 100% sequence identity.

The presence of at least two major evolutionary lineages of *S. scabiei* occurring among wombats in Tasmania, and wombats and koala mites from Victoria suggests a polyphyletic origin may exist for *S. scabiei* in Australia. The absence of major clade separation between wombats and koala mites suggests a level of pathogen generality, indicative of multiple cross-species transmission events. Although we did not sample other animals in this study, we think it is reasonable to hypothesise that these cross-species transmission events may also include other animals such as domestic and wild dogs and invasive foxes.

Although more sampling is clearly required to address this question conclusively, our observations supports the hypothesis that *S. scabiei* was first introduced to Australian wildlife in the time following European colonisation. This is based on (i) significant sequence similarity in the NFL mitochondrial genomes of the marsupial mites sampled in this study and; (ii) the single gene molecular typing results that reveal that *cox1* haplotypes are shared between marsupial mites and those from a diverse range of animal hosts in the rest of the world, Indeed this is not a new suggestion [59, 82, 102, 128], but our data provides the strongest support yet for this hypothesis. Historical records of mange disease in humans and domestic dogs from Tasmania date back to as early as the 1820s [137, 138], suggesting that Australian wildlife have been infested by *S. scabiei* for at least 200 years. The main question appears to simply be how long before then were mites introduced. Pre-European hypotheses must still be considered (albeit weakly) plausible, including introduction through aboriginal colonisation (ca. 50,000–60,000 years ago), introduction of the dingo (ca 4000 years ago), or deeper evolutionary origins. If *S. scabiei* mitochondrial genome evolutionary rates are similar to that of other invertebrates (i.e. 10−6 substitutions per year [139] [140]), then the evolutionary timescale of Australian

marsupial mite divergence would be likely greater than >200 years. This prediction, however, does not take into account potential changes to this rate associated with spill-over into a new host and the obvious limitations of making these interpretations on such a small sample set. Although a less parsimonious explanation, we also cannot obviously exclude the potential for mitochondrial capture and/or selective sweep phenomena to potentially mask the genetic signature of mites that may have been present in Australian animals prior to European colonisation. The resolution of the origins of *S. scabiei* in Australia will require sampling of mites from a greater time span from humans, marsupials and other hosts or a significant expansion in genomic sequence data for *S. scabiei*.

The availability of an extended set of mitochondrial sequences beyond the first initial human *S. scabiei* mitochondrial genome sequence provided an opportunity to re-assess the suitability of different mitochondrial genetic markers for detailed molecular epidemiology. Our research confirms that the two mitochondrial rRNA genes (12S and 16S) which have been used to infer origins of *S. scabiei* to Australia in the past, provide insufficient resolution for such assessments. The 16S rRNA gene, which is the more variable of the two genes (eight haplotypes identified in this study), can differentiate mites from differing host families, but cannot separate host species and location specific *S. scabiei* [128, 129], or preserve the resolution of ancestral origin when compared to the NFL mitochondrial genome. It has been suggested that *cox1* should be used as mitochondrial genetic marker due to the high variability and its ability to distinguish between biogeographical regions, host families, genera, species and even subspecies [128, 129, 141, 142]. Here, we also support this suggestion, with the phylogenetic reconstruction of the gene preserving an identical tree to the NFL mitochondrial genome. In the absence of available NFL genetic data for downstream molecular epidemiological investigations of *S. scabiei* in humans and animals, our analysis would appear to support the continued use of *cox1* as a gene target for molecular typing.

To explore this further, we combined the new *cox1* sequences from this study with new haplotypes from Europe [130] and other global haplotypes [128] to interrogate phylogenetic associations among hosts globally. Confirming our previous research [128], we found the Australian *S. scabiei* haplotypes share sequence identity with mites from non-human hosts in Europe, the Middle-East and Asia. These multiple geographic associations among a variety of host species potentially supporting multiple introduction events of *S. scabiei* from differing geographic regions into Australia, as indicated by the two mitochondrial genome clades shown in this study and the presence of multiple genetically distinct mites in Tasmania, some of which are also found on the mainland. Cross-host transmission has previously been demonstrated in the case of wombat to human transmission [62] and other experimental cross-infestations [87, 103, 143]. While clear genetic evidence for cross-host transmission between Australian marsupials and other hosts is lacking at this stage, the observation of shared *cox1* gene sequences (and similar NFL mitochondrial genome sequence data from this study) between Australian marsupial species suggests this is very likely. This observation alone highlights the need for additional precautions in the management of mange between different Australian wildlife and a renewed emphasis on infection control practices for stakeholders involved in their care.

3.6 Conclusions

This study is the first to sequence the mitochondrial genomes of *S. scabiei* in Australian marsupials. In doing so, we revealed a high level of sequence similarity among the marsupial mite sequences supporting the likely transmission of these mites between marsupial hosts affected. A greater repertoire of mites from more marsupial hosts spanning across Australia and other hosts globally would be beneficial. Mites from dingos, foxes and wild dogs in Australia would be particularly ideal as they likely contribute to the intra- and inter-specific transmission dynamics of *S. scabiei* on mainland Australia. Furthermore, an expansion of

sample collection over time and geographical areas would also be beneficial for estimating rates of evolution, allowing evolutionary divergence to be better assessed. Our comparison of gene trees confirmed the use of *cox1* as the most informative gene (when sequenced in full length) for phylogenetic comparisons. An expansion of such studies leveraging the genetic data provided in this study is expected to provide further insight into the global dissemination of this widespread and neglected human and animal pathogen.

CHAPTER 4

EXPANDED MOLECULAR TYPING OF *S. SCABIEI* PROVIDES FURTHER EVIDENCE OF DISEASE SPILL-OVER EVENTS IN THE EPIDEMIOLOGY OF SARCOPTIC MANGE IN AUSTRALIAN MARSUPIALS

Tamieka A Fraser[a,b], Roz Holme[c], Alynn Martin[a], Pam Whiteley[d], Merridy Montarello[e], Cam Raw[d], Scott Carver[a], Adam Polkinghorne[b*]

[a] Department of Biological Sciences, University of Tasmania, Sandy Bay, Tasmania, Australia

[b] USC Animal Research Centre, School of Science and Engineering, University of the Sunshine Coast, Sippy Downs, Queensland, Australia

[c] Cedar Creek Wombat Rescue Inc. & Hospital, Cedar Creek, New South Wales, Australia

[d] Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Werribee, Victoria, Australia

[e] Fauna Rescue SA Inc., Netherby, South Australia, Australia

This paper was accepted for publication as a short communication in Journal of Wildlife Diseases; 13th June 2018.

Statement of joint authorship

Conceived and designed experiments: TF, AP, SC. Collected samples: PM, AM, RH, MM, CR and TF. Phylogenetic analysis: TF. TAF wrote this manuscript with drafting contributions and approval from remaining authors.

4.1 Abstract

The invasive ectoparasite *Sarcoptes scabiei* impacts on the welfare and conservation of Australian marsupials. Previous molecular data has suggested that spill-over from other hosts may be responsible for the emergence of this infectious disease, however, the scale of such studies is sample limited. We performed expanded molecular typing of the *S. scabiei* mitochondrial *cox1* gene from 81 skin scrapings from infested wombats, koalas, foxes and dogs across Australia. Combined with existing *S. scabiei* sequences, our analysis revealed 16 haplotypes amongst Australian animals, sharing between 93.3% and 99.7% sequence similarity. While some sequences were unique to specific hosts and/or to Australia, key haplotypes could be detected across several marsupial hosts as well as wild or domestic canids in Australia. In a global analysis, we identified 43 *cox1* haplotypes with many Australian haplotypes identical to *S. scabiei* mites from inside and outside Europe. On this basis, we conclude that multiple introduction events are plausible explanations to the origin and emergence of this parasite into Australian marsupials and that disease spill-over from canids is likely. Together, our greatly expanded *S. scabiei* sequence dataset provides a more nuanced picture of both spill-over and sustained intra-specific transmission for this important parasite.

Keywords: scabies, molecular typing, One Health, epidemiology

4.2 Background

Sarcoptes scabiei is an emerging invasive ectoparasite known to infest over 100 mammal species globally, including humans [5, 20]. Signs of sarcoptic mange (classified as scabies in

human) include severe dermatitis, alopecia, pruritus, and hyperkeratosis. Septicaemia and death may then eventuate [20]. High mortality rates of up to 90% have been reported in a variety of European wild mammals [144, 145].

In Australia, sarcoptic mange is reported in a range of native and feral mammals, as well as humans and domestic animals [128]. Wombats appear to be particularly susceptible with a recent report describing an epizootic leading to a 94% decline in a bare-nosed wombat (*Vombatus ursinus*) population [38]. The emergence of sarcoptic mange in geographically isolated populations of Australian marsupials is potentially explained by growing evidence that *S. scabiei* may have been introduced into Australia by European settlement in the 1800s [34]. The first evidence supporting this arose from analysis of partial gene sequences from a small collection of marsupial *S. scabiei* samples, revealing close similarity to sequences from domestic animals and humans [72, 82]. Whole mitochondrial genome sequencing of several *S. scabiei* mites from koalas and wombats confirmed these earlier observations, detecting genetically distinct *S. scabiei* mitochondrial DNA sequences in marsupials with some sharing closer genetic relationships to mites from hosts in other countries than to other marsupial *S. scabiei* sequences [146]. While providing further insight into the potential evolutionary history of this parasite in Australian marsupials, extensive population genetics data for *S. scabiei* is still lacking to improve inference about spill-over events and the potential sources of the infestation. In the current study, we begin the process of establishing a population genetic framework for *S. scabiei* in Australian animals, acquiring new mite sequences from a variety of hosts including dogs, foxes, koalas and bare-nosed wombats across five states of Australia.

4.3 Methods

A total of 81 skin scrapings were collected from four Northern Territory (NT) dogs, four South Australian (SA) koalas, two Victorian (VIC) foxes, and 11 Tasmanian (TAS) and 45

New South Wales (NSW) bare-nosed wombats across several geographic locations. Collection of skin scrapings during routine veterinary diagnosis of mange-infested animals was approved by the Animal Research Committees at the University of the Sunshine Coast (approval AN5/16/43) and University of Tasmania (approval A0014670) and state permits from the Office of Environment & Heritage NSW National Parks & wildlife Service (SL101719), Department of Primary Industries, Park, Water and Environment for Tasmania (approval FA15121) and Victorian Department of Environment and Primary Industries (10007943).

Scrapings were stored in 100% ethanol. DNA extractions were performed on both pooled mites from a single skin scraping or on the complete skin scraping using a QIAamp DNA mini kit (Qiagen, Hilden, Germany). Conventional PCR and sequencing, targeting a 400bp fragment of the *cox1* gene [67], was then performed. This gene was selected for molecular typing since previous analyses showed it was phylogenetically informative and mostly congruent with whole *S. scabiei* mitochondrial genome phylogenies [128, 146]. Each 25µL conventional PCR assay consisted of 1 X Amplitaq Gold 360 Master Mix (Life Technologies), 0.3 µM of forward and reverse primers and 5µL DNA template. Cycling conditions were 95°C for 10mins, 35 cycles of 95°C for 15s, 51°C for 30s and 72°C for 1 min, followed by a final extension of 72°C for 7 mins. Positivity was confirmed following agarose gel electrophoresis and UV transillumination. Amplicon sequenced was performed by Macrogen Inc.

All sequences were aligned with ClustalW and phylogenetic tree construction was performed by Mr. Bayes, rooted with *Otodectes cynotis* (KP676688) with 1000 bootstrap replicates, implemented by Geneious 9.1.8 [131]. Phylogenetic meta data analysis was performed with Phandango [147].

Table 4.1: Detailed list of Australian *S. scabiei cox1* sequence haplotypes and their relationship to sequences detected in other parts of the world.

Haplotype	Australian Host	Host Location	Accession Numbers	Identical to Global Sequences
1	Dog	Northern Territory	AY493394	N/A
2	Wallaby	South Australia	AY493398	Dog: KT961029 (France)
	Wombat	Victoria	AY493397, MF083739	Dog: KT961025 (Italy)
	Dog (N= 1)	Northern Territory	MH104877*	Dog: AY493393 (USA)
				Dog: KT961022 (China) Fox: KT961032, KT961030, KT961031 (France)
3	Dog	Northern Territory	AY493392	N/A
4	Dog	Northern Territory	AY493391	N/A
	Wombat	Victoria	MF083738	
	Koala	Victoria	MF083734, MF083742	
	Koala (N= 4)	South Australia	MH104878*	
	Fox (N= 2)	Victoria		
	Dog (N= 3)	Northern Territory		
5	Wombat	Tasmania	MF083741, MF083740	N/A
	Wombat (N= 11)	Tasmania	MH104879*	
6	Human	Northern Territory	AY493383	N/A
7	Human	Northern Territory	LN874268, AY493382	N/A
8	Human	Northern Territory	AY493384	N/A
9	Koala	Victoria	MF083732, MF083733, MF083743	Dog: KJ499544 (China)
	Wombat	Tasmania	MF083737	Dog: AB821011 (Japan)
	Wombat (N= 5)	New South Wales	MH104880*	Racoon Dog: AB821007, AB821012, AB821004 (Japan)
10	Wombat (N= 1)	New South Wales	MH104881*	N/A

11	Wombat (N= 1)	New South Wales	MH104882*	N/A
12	Wombat Wombat (N= 43)	New South Wales New South Wales	MF083735, MF083736 MH104883*	Sheep: AB779609, AB779608 (Egypt) Buffalo: AB779594 (Egypt) Jackal: KP987792 (Israel)
13	Wombat (N= 1)	New South Wales	MH104884*	N/A
14	Wombat (N= 2)	New South Wales	MH104885*	Dog: KT961026 (South Africa) Dog: KT961028 (China) Dog: KT961024 (France)
15	Human	Northern Territory	AY493388, AY493385	Human: KR058190 (France)

* Haplotype accession numbers identified and described by the current study

4.4 Results and Discussion

PCR amplification and *cox1* gene sequencing resulted in 81 new Australian-derived *S. scabiei* sequences to add to analyses of 24 previously described sequences (Table 4.1). Sixteen unique *cox1* haplotypes could be identified in the Australian hosts following *cox1* sequence alignment, sharing between 93.3% and 99.7% sequencing similarity. These haplotypes could be broadly divided into five groups (Table 4.1) including (i) three novel *S. scabiei cox1* haplotypes (H10, H11 & H16) in wombats from NSW; (ii) two Australian *S. scabiei cox1* haplotypes (H4, H5), representing *S. scabiei* sequences from NT dogs and TAS wombats and identical to *S. scabiei* sequences previously only observed in other Australian animals; (iii) four *S. scabiei* haplotypes (H2, H9, H12 & H14), representing sequences from dogs and wombats, that were identical to previously described sequences from Australian marsupials (wallabies, wombats) and dogs as well as hosts from other parts of the world; (iv) haplotypes detected in humans in Australia and other parts of the world (H6, H7, H8, H15 &16); and (v) two Australian-specific dog haplotypes (H1 & H3) previously identified by Walton et al. [72].

To understand the distribution and relationships of these sequences further, we constructed a phylogenetic tree of all Australian available *cox1* sequences (Fig. 4.1). In the case of bare-nosed wombats, the most comprehensively sampled host in this study, at least nine genetically distinct *S. scabiei* strains have now been detected revealing an unexpectedly high level of genetic diversity of circulating *S. scabiei*. The NSW wombat population appears to harbour at least six *S. scabiei* haplotypes (H9 – H14) with three sequences unique to this population (H10, H11, H13), while the other three have been detected in other hosts globally (H12, H14) and/or in other marsupials in other states (H9) (Table 4.1). The observation that many of these 'wombat' haplotypes could also be detected in koalas (indeed, we did not detect any koala-specific lineages), suggests that individual lineages of *S. scabiei* mites can readily infest a range of Australian marsupials.

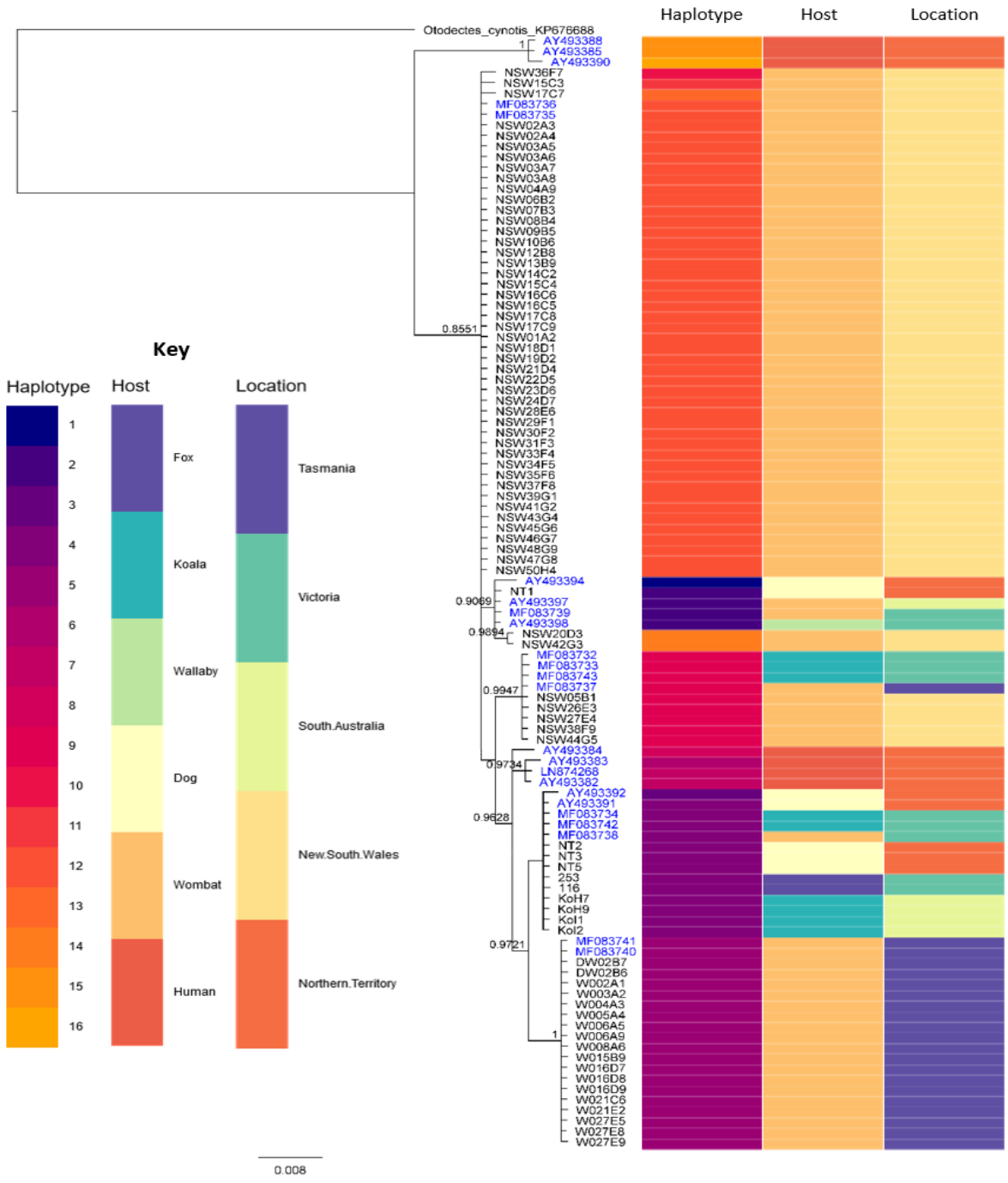

Figure 4.1: Phylogenetic tree of Australian *S. scabiei cox1* sequences. Bayesian phylogenetic analysis of 105 cox1 sequences for Australian hosts only, rooted with *Otodectes cynotis*. Nodes highlighted in blue represent previously available sequences from GenBank, while black labels indicate sequences obtained in the current study. Bootstrap values >80 are indicated. Haplotype, host and location are colour coded, corresponding to each node label using Phandango [147].

In terms of evidence for disease spill-over in Australia, we also detected at least one *S. scabiei* *cox1* haplotype (H4) that was previously detected in dogs from the NT and, in our study, was shared by VIC wombats, koalas and foxes. This same haplotype could also be detected in SA koalas and, again, from dogs more recently sampled in the NT. Since this appears to be an Australian-specific lineage, we cannot yet deduce the directionality of this cross-host transmission, however, dogs have long been suspected as a potential source of marsupial *S. scabiei* infestation [82] and this new data, including the identification of this same sequence in foxes, supports this hypothesis. Perhaps an even better case for canine reservoirs of marsupial infestation also lies in the detection of *cox1* haplotype 2 (Table 4.1) in a dog in this study and marsupials from previous studies [72, 146], as well as a range of other dogs in the rest of the world.

The failure to separate the majority of Australian *S. scabiei* sequences from other global sequences, as well as the presence of at least one human-specific lineage (containing haplotypes H15 and H16), is further illustrated in Fig. 4.2. Together this data supports a hypothesis that Australian marsupials may be a 'sink' for genetically diverse animal *S. scabiei* lineages introduced from the rest of the world, additionally evidenced by haplotypes H9, H12 and H14 which include domestic and non-domestic animals from outside Australia (Table 4.1). This hypothesis of multiple mange introduction events has also been implicated in North American bears [43] and canids in Japan [148].

This is the largest scale phylogenetic assessment of sarcoptic mange infesting Australian animals and provides a basis for building a more detailed population genetic framework of *S. scabiei* across all Australian animal populations. This analysis revealed a high level of *S. scabiei* genetic diversity within a given animal population. Whether this is the norm for this parasite is unclear because similar size studies are very limited. Based on the evidence in this study, previous studies of mange in Australian animals [146] and studies from other wildlife.

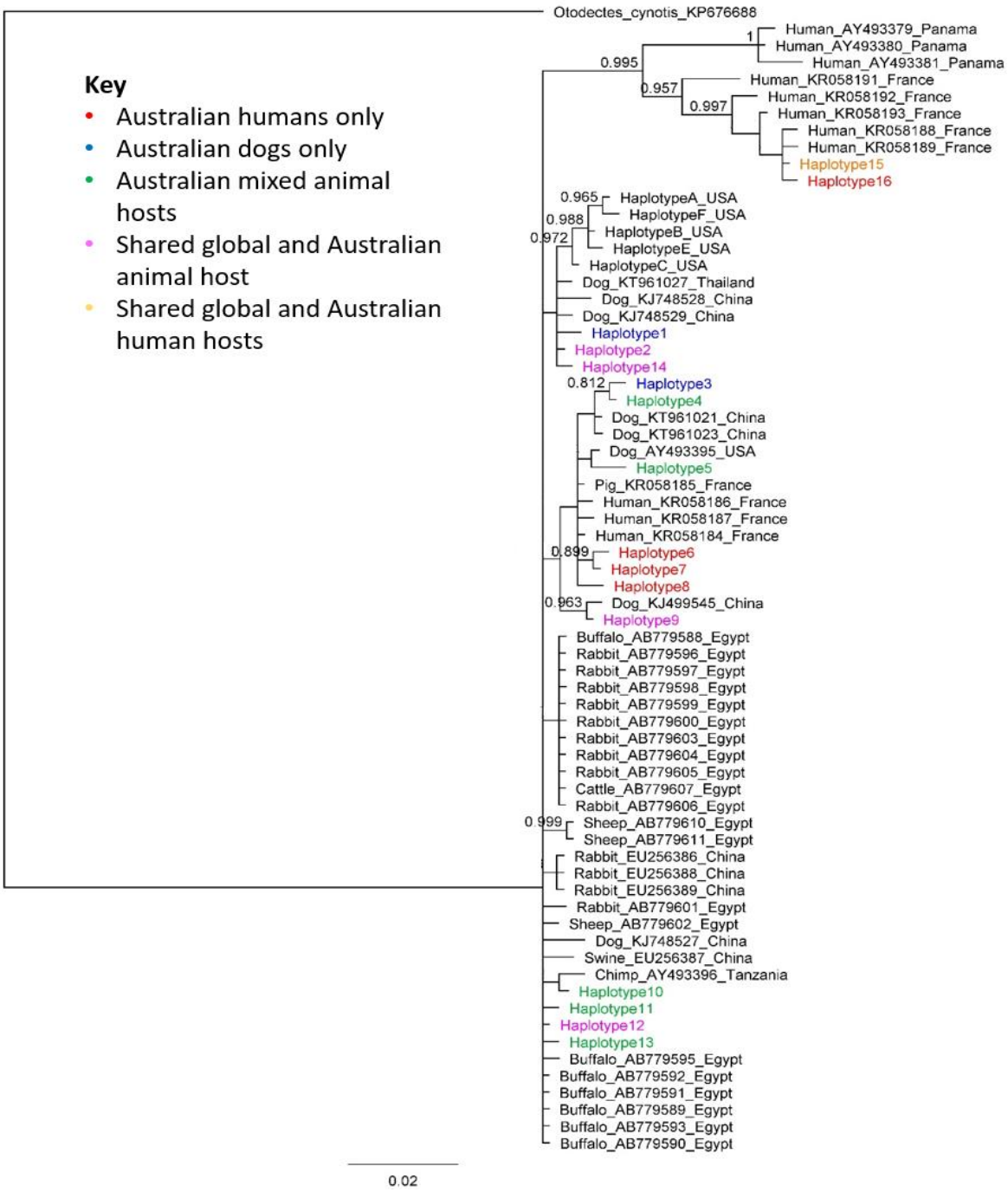

Figure 4.2: Global Bayesian phylogenetic analysis for the *cox1* gene of *S. scabiei*. Phylogenetic assessment of Australian haplotypes and globally available *cox1* sequences for *S. scabiei*, rooted with *Otodectes cynotis*. Bootstrap values >80 are shown. Haplotypes for (i) Australian humans only, (ii) Australian dogs only, (iii) Australian animals only, (iv) shared global and Australian animal hosts and, (v) shared global and Australian human hosts are indicated by colour key.

hosts [43], what is clear is that some of this genetic diversity is likely due to multiple introductions of *S. scabiei* via canids (dogs and foxes) and possibly humans. More detailed sympatric sampling studies will be required to resolve this question.

CHAPTER 5

COMPARATIVE DIAGNOSTICS REVEALS PCR ASSAYS ON SKIN SCRAPINGS IS THE MOST RELIABLE METHOD TO DETECT *SARCOPTES SCABIEI* INFESTATIONS

Tamieka A. Fraser[a,b,*], Alynn Martin[a], Adam Polkinghorne[b], Scott Carver[a]

[a] School of Biological Sciences, University of Tasmania, Sandy Bay, 7001, TAS, Australia

[b] Centre for Animal Health Innovation, University of the Sunshine Coast, 91 Sippy Downs Drive, Sippy Downs, 4556, QLD, Australia

This paper was published in Veterinary Parasitology, 2018. 251: p. 119-124

Statement of joint authorship

TF, SC and AP conceptualised the manuscript. Samples were collected by TAF and AM. Experiments and analysis was conducted by TAF. TAF wrote this manuscript with drafting contributions and approval from remaining authors.

5.1 Abstract

Sarcoptic mange is a globally significant parasitic disease of humans and other animals, both domestic and wild. But clinical diagnosis of *S. scabiei* infestation, using the standard skin scraping followed by microscopy technique, remains highly variable (predominantly due to false-negatives), and a major challenge for human and animal welfare. Here, we utilised a unique sample set from bare-nosed wombats (*Vombatus ursinus*) to evaluate a variety of putatively useful diagnostic approaches for *S. scabiei*. Against the standard of skin scrapings followed by microscopy, we compared observational scoring of mange severity (often employed in field studies of wildlife), PCR on skin scrapings (recently proposed as an improvement for humans and other animals), and PCR on skin swabs (proposed a non-invasive method for humans and other animals). We find that observational scoring positively correlated with counts of *S. scabiei* from skin scrapings, particularly as mange severity increases, but underdiagnoses early mange. Species-specific PCR for *S. scabiei* on skin scrapings had enhanced capacity for mite detection relative to microscopy. Finally, the non-invasive sampling method of PCR on skin swab samples had a high congruence to skin scraping microscopy, however prospective false negatives as a consequence to sampling is concerning. To our knowledge, this is the first study to simultaneously assess this combination of methods for *S. scabiei* diagnosis. We conclude that PCR on skin scrapings as an advancement on traditional microscopy, and the other techniques (observational, skin swabs and microscopy) remain useful, but harbour greater false-negatives. Outcomes are transferrable to diagnosis of *S. scabiei* for other host species, including humans, particularly for crusted mange and potentially ordinary mange also.

Keywords: Sarcoptic mange, *Sarcoptes scabiei*, Diagnosis, Diagnostic technique, One health

5.2 Introduction

The ectoparasitic mite *Sarcoptes scabiei*, is a cause of significant human and animal welfare concern, and economic burden, globally [14, 23, 87]. This parasite causes>300 million human cases of scabies per year and has been documented to cause mange in>100 species of mammals [5, 149, 150]. Pathology results from burrowing of the mite into the skin, causing irritation, inflammation, alopecia, pruritis, lesions and hyperkeratosis [20]. Two forms of disease have been described, ordinary and crusted. Ordinary scabies, the more common manifestation, results in pruritic skin lesions with mite infestations typically averaging between 5 and 15 mites per affected individual [22]. Crusted scabies, on the other hand, is a more severe and rare manifestation with thousands to millions of mites infesting an individual [19].

Despite its significance, clinical diagnosis of *S. scabiei* infestation in humans and other animals remains a challenge. Classical diagnosis involves the process of skin lesion examination and skin scraping the epidermis for visualisation of the mite and/or eggs by microscopy [36]. This technique is commonly used by both medical practitioners for humans and veterinarians for other animals, as *S. scabiei* has a relatively distinct morphology. The level of diagnostic sensitivity using this method is well known to be variable, owing to low numbers of mites in early stages of disease and, more generally, during ordinary scabies [19, 34]. Even with crusted scabies, the ability to obtain mites from scrapings of thickened skin can be problematic [19].

While molecular techniques have been applied to studying the molecular epidemiology of *S. scabiei* infections in humans and animals [14, 128], their use as an ancillary diagnostic tool for detection of *S. scabiei* DNA is relatively new. A recent study used nucleic acid amplification by conventional and quantitative PCR and reported both methods to have a higher sensitivity for mite identification over microscopy in human scabies skin scrapings [151]. Interestingly, analysis of swab samples by PCR collected from the crusted scabies patients [151] showed

comparable results to skin scrapings, raising this as an alternative and less invasive method for collection of a scabies diagnostic specimen. Additionally, diagnosis of sarcoptic mange in non-human animals is often made by observation in field setting. Observational scoring systems are based on signs of alopecia, epidermal inflammation, hyperkeratosis and crusting, and have been used in a range of wildlife, including wolves [37], coyotes [39], chamois [41], and wombats [38, 40]. Scoring systems assess the severity of mange disease, but are rarely evaluated against actual counts of mites.

In this study, we evaluate diagnostic methodologies for *S. scabiei* infestation. Focussing on bare-nosed wombats (*Vombatus ursinus*), we exploit a small (n=23), but rare sample set that enables comparison of PCR on skin scrapings and swabs, and observational scoring of mange severity, to classical *S. scabiei* diagnosis based on microscopy of skin scrapings. We evaluate (a) the sensitivity and specificity of each method against microscopy of skin scrapings, and (b) the relationship between observational scoring of mange severity to mite burden. To the best of our knowledge, this is the only study to simultaneously evaluate this spectrum of *S. scabiei* diagnostic approaches.

5.3 Methods

5.3.1 Study system and sample collection

Within Australia, *S. scabiei* is known to infest humans, domestic and invasive mammals, and native wildlife, causing health, welfare and conservation concerns [5, 20, 74]. Bare-nosed wombats (*Vombatus ursinus*) are the most impacted wildlife species. A total of 23 bare-nosed wombats were caught, anesthetised, assessed using a mange scoring system (Simpson et al., 2016) and subjected to skin scrapings and swab collection (Fig. 5.1) during a mange epizootic at Narawntapu National Park, Tasmania (University of Tasmania Animal Ethics No. A0014670).

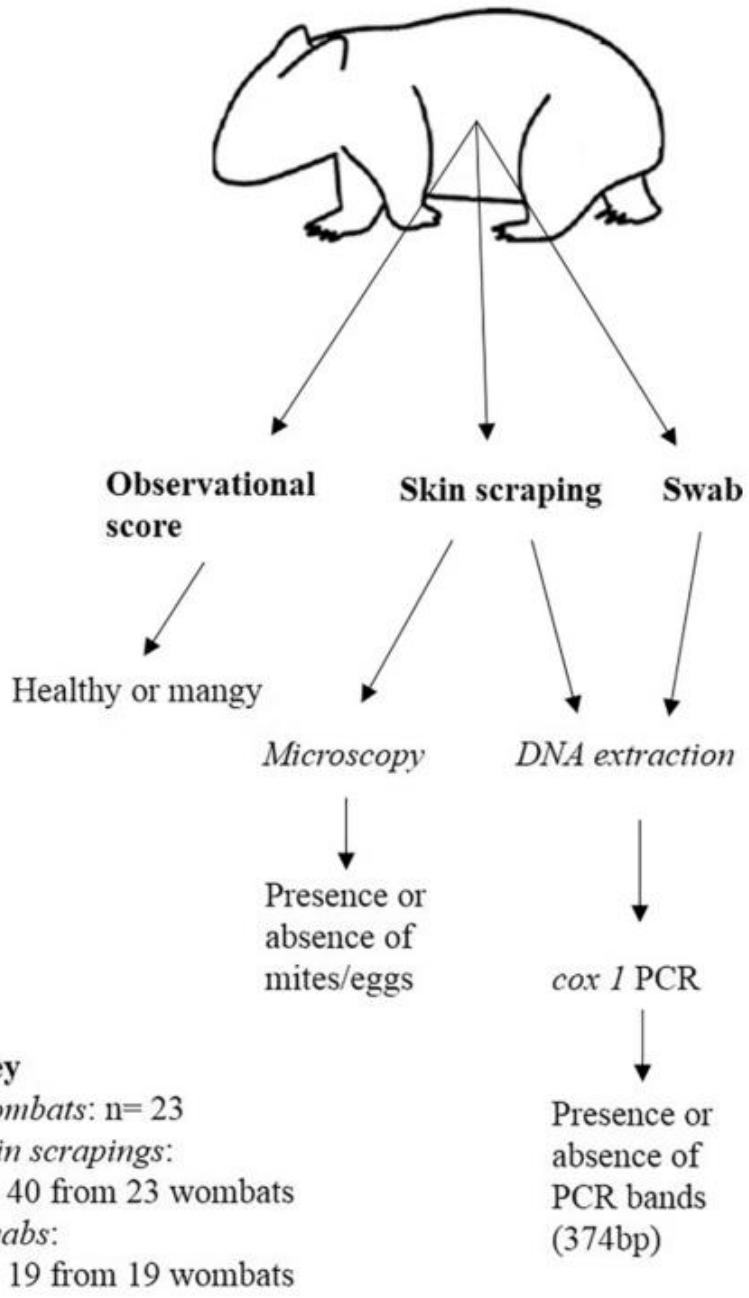

Figure 5.1: Samples collected for each wombat for different diagnosis methods of sarcoptic mange. Three types of samples were collected for each wombat consisting of a mange score for each body segment, a skin scraping and a swab. A total of 40 skin scrapings were obtained from 23 wombats, and only 19 swabs were collected from 19 wombats.

The observational scoring system of mange in wombats is based on dividing each side of the wombat body into 14 different segments and scoring each segment on a 1–10 scale [40]. Wombats were classified as healthy if all body segments had a mange score of 0–2, and mange infested if any body segment had a score>2 [38, 40]. Mange severity for individual wombats was classified by the highest body segment mange score with scores of 3 as early mange, 4–6 as moderate mange, 7–8 as severe mange and 9–10 as late stage mange [38].

Dry skin scrapings were taken using routine methodology and independently assessed by a veterinarian at the time of collection. Briefly, the skin of an affected area of the wombat was pinched firmly and, at a 90° angle to the skin, a scalpel blade was used to remove skin cells prior to capillary breakage for a 1–2cm2 area. Cell debris and any *S. scabiei* mites collected were then placed into Eppendorf tubes containing 100% ethanol for future microscopy analysis. The flank of the wombat was preferentially sampled in all cases, and additional sites were sought on 10/23 individuals opportunistically. Additionally, Catch-All™ sample collection swabs (Gene Target Solutions) were moistened with saline before application to the skin in a firm rotatory motion in a 1 cm by 2 cm area. Swabs were taken from the flank of the wombat. All skin scrapings and swabs were stored at −20 °C for future analysis.

5.3.2 Identification by microscopy

Identification of the *S. scabiei* mite from skin scrapings requires visualisation of the mite and/or eggs [152]. For this purpose, skin scrapings stored in ethanol were emptied into a petri dish for inspection under a dissection microscope. Mite counts were recorded for each skin scraping individually. Skin scraping samples were then returned to the Eppendorf prior to DNA extractions. At no point were samples allowed to desiccate completely or have ethanol evaporate substantially away during this process.

5.3.3 DNA extractions

To extract mite DNA from collected swabs, a DNA extraction was performed using a QIAamp DNA mini kit (Qiagen, Hilden, Germany), with minor modifications to the user-developed protocol (Qiagen, Hilden, Germany). Briefly, 300 µL of ATL buffer was added to the swab and vortexed for 3 min to shake off any adhered cells and mites. The swab was removed after vortexing and this solution was heated at 56 °C overnight with the addition of 40 µL of proteinase K. Purification steps were completed as per the manufacturer instructions and final DNA was eluted into 100 µL of the supplied AE buffer (Qiagen, Hilden, Germany). Skin scrapings stored in 100% ethanol were spun at 17,000×g for 5 min to pellet skin debris and any mites/eggs. Ethanol was pipetted off and the pellet was air-dried at 56 °C to remove any excess ethanol prior to DNA extractions. DNA extractions were completed for these skin scrapings using the above method without the 3 min of vigorous shaking.

5.3.4 *S. scabiei* cox1 and β-actin PCR

Specific *S. scabiei* primers were designed to amplify a 374 base pair (bp) fragment of the cox1 gene for the purpose of detection of *S. scabiei* in both swab and skin scraping samples. This was designed from the alignment of new Australian marsupials [146] and the human [119] *S. scabiei* mitochondrial genomes (Fig. 5.2). Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi#) and OligoAnalyzer 3.1 online tool (https://sg.idtdna.com/calc/analyzer) were used to analyse primers for DNA base mismatches. The total volume of each PCR assay was 25 µL consisting of 1 X Amplitaq Gold 360 Master Mix (Life Technologies), 0.3 µM of forward (5′CTGGTAGAGGAACTGGCTG3′) and reverse (5′GTAAACTTCCGGGTGTCC3′) primers and 5 µL DNA template. Cycling conditions for the thermocycler were 95 °C for 10mins, 35 cycles of 95 °C for 15 s, 58 °C for 30 s and 72 °C for 1 min, followed by a final extension of 72 °C for 7 mins. Each reaction was visually verified

Figure 5.2: Alignment of cox1 fragment from available mitochondrial genomes indicating primers and the haplotype derived from the 374bp PCR sequencing. All samples sequenced in this study produced the same haplotype. Forward primer sits at positions 2810-2828 and reverse primer between 3166-3138 of the full length *cox1* gene in the human strain LN874268.

by ethidium bromide on a 1.8% TBE agarose gel under UV. Sequencing PCR amplicons by Macrogen Inc. was performed on samples as well as mite DNA for validation of primer specificity. Chromatograms confirmed *S. scabiei* cox1 sequence of the correct length as indicated in Fig. 5.2. Detection limit of this cox1 PCR was assessed by a serial dilution of known PCR purified product from $10^{\wedge 9}$ to $10^{\wedge 0}$ copies of the 374 bp cox1 fragment. By gel visualisation, we were able to detect 100 copies.

Vertebrate primers to amplify a 110 bp fragment of β-actin [153] were used to assess the quality of samples where *S. scabiei* could not be detected. Assays consisted of 2 X Quantitect (Qiagen), 0.25 μM forward and reverse primers and 5 μL of DNA template. Real time PCR conditions were 95 °C for 15mins, 35 cycles of 94 °C for 15s, 60 °C for 30 s and 72 °C for 30 s, and melt curve between 65 °C and 95 °C.

5.3.5 Statistical comparisons

In each case, the sensitivity, specificity and Cohen's kappa coefficient for inter-rated agreements of mange score, scraping PCR and swab PCR were made against the clinical diagnostic tool of microscopy using diagnostic and agreement statistics [154]. Additionally, to compare the relationship between mange severity score with the number of mites obtained by skin scrapings, Spearman correlation analysis was conducted using the statistical program, R [155].

5.4 Results

A total of 23 bare-nosed wombats inhabiting Narawntapu National Park, Tasmania, were sampled for this study. Observational scoring of mange severity was made on all wombats with a total of 40 skin scrapings taken (Table 5.1). Of the 23 wombats, 10 had multiple (2–3) skin scrapings taken from different body sites. For individual wombats deemed healthy or with early mange, scraping sites were chosen that we considered likely to maximise the probability of

Table 5.1: Observational classification of *S. scabiei* infestation severity and skin scraping with microscopy outcomes.

Wombat ID	Mange Classification	Body Segment Skin Scraping (Mange Score: Mite Tally)
DW02	Severe	Flank (10: 11)
W002	Healthy	Left Flank (1: 0), Right Flank (1: 2)
W003	Severe	Flank (10: 13)
W004	Moderate	Flank (5: 0), Neck (3: 2)
W005	Severe	Flank (3: 13)
W006	Moderate	Flank (3: 2), Posterior Limb (5: 0)
W008	Early	Flank (1: 8)
W009	Healthy	Flank (0: 0)
W010	Healthy	Flank (0: 0)
W013	Healthy	Flank (0: 0), Anterior Limb (1: 0)
W014	Healthy	Flank (0: 0), Posterior Limb (1: 0)
W015	Healthy	Anterior Limb (0: 0)
W016	Early	Flank (2: 1), Posterior Limb (1: 4), Neck (1: 7)
W017	Healthy	Flank (0: 0), Posterior Limb (0: 0)
W018	Healthy	Flank (0: 0)
W019	Healthy	Flank (0: 0), Anterior Limb (1: 0)
W020	Healthy	Flank (0: 0)
W021	Severe	Flank (2: 7), Posterior Limb (7: 11)
W022	Healthy	Posterior Limb (1: 0)
W023	Healthy	Posterior Limb (1: 0), Posterior Limb (0: 0), Shoulder (0: 0)
W024	Healthy	Flank (0, 0-0)
W025	Early	Flank (2, 0-0-0)
W027	Early	Flank (3: 3, 1, 0)

Microscopy results of 40 skin scrapings taken from 23 wombats, with mange score and mite tally for a particular skin scraping segment. Individual wombat overall mange classification/status was classified as the highest body segment having a score of 3 (early mange), 4-6 (moderate mange), 7-8 (severe mange) and 9-10 (late stage). Healthy wombats were classified by having the highest body segment with a mange score of 0-2.

mite sampling. Three wombats had repeat skin scrapings taken from the same anatomical site (Table 5.1). A total of 19 wombats had swab samples collected from the flank, corresponding to skin scraping obtained.

5.4.1 Observational scoring shows moderate agreement to microscopy outcomes

Using the previously described mange scoring scheme for wombats [38], 13/23 (56.52%) were classified as healthy (highest body segment score ≤2) with seven of these animals receiving all body segments with a mange score of 0. For those animals that received a mange score of >2 (10/23), four were classified as having signs of early mange (highest body segment score =3), two with moderate mange (highest body segment score 4–6), and four at late stage mange (highest body segment score 9–10). No animal was recorded with severe mange (highest body segment score 7–8), according to the scoring scheme.

Following microscopic examination, 10/23 wombats were found to have one or more detectable mites in their skin scrapings (Table 5.1). For body segments that were classified as healthy (mange score ≤2), 6/23 (26.09%) were positive for mites microscopically. For individual body segments that were classified as mangy (body segment score >2), 7/9 (77.78%) were positive for mites. Three wombats had two or three skin scrapings taken from the same body segment (Table 5.1; W024, W025 and W027). W024 and W025 were classified as healthy and early, respectively, based on mange score, and an absence of mites was confirmed by the repeated skin scrapings. Wombat W027 had a mange score of 3, therefore classified as mangy, and microscopy analysis identified three, one and zero mites for this individual's three individual skin scrapings (Table 5.1).

When the body segment score was compared with mite positivity and load, Spearman's correlation analysis revealed a significant positive association between body segment mange score and mite identification (ρ=0.60, P< 0.001, Fig. 5.3). Sensitivity of observational scoring

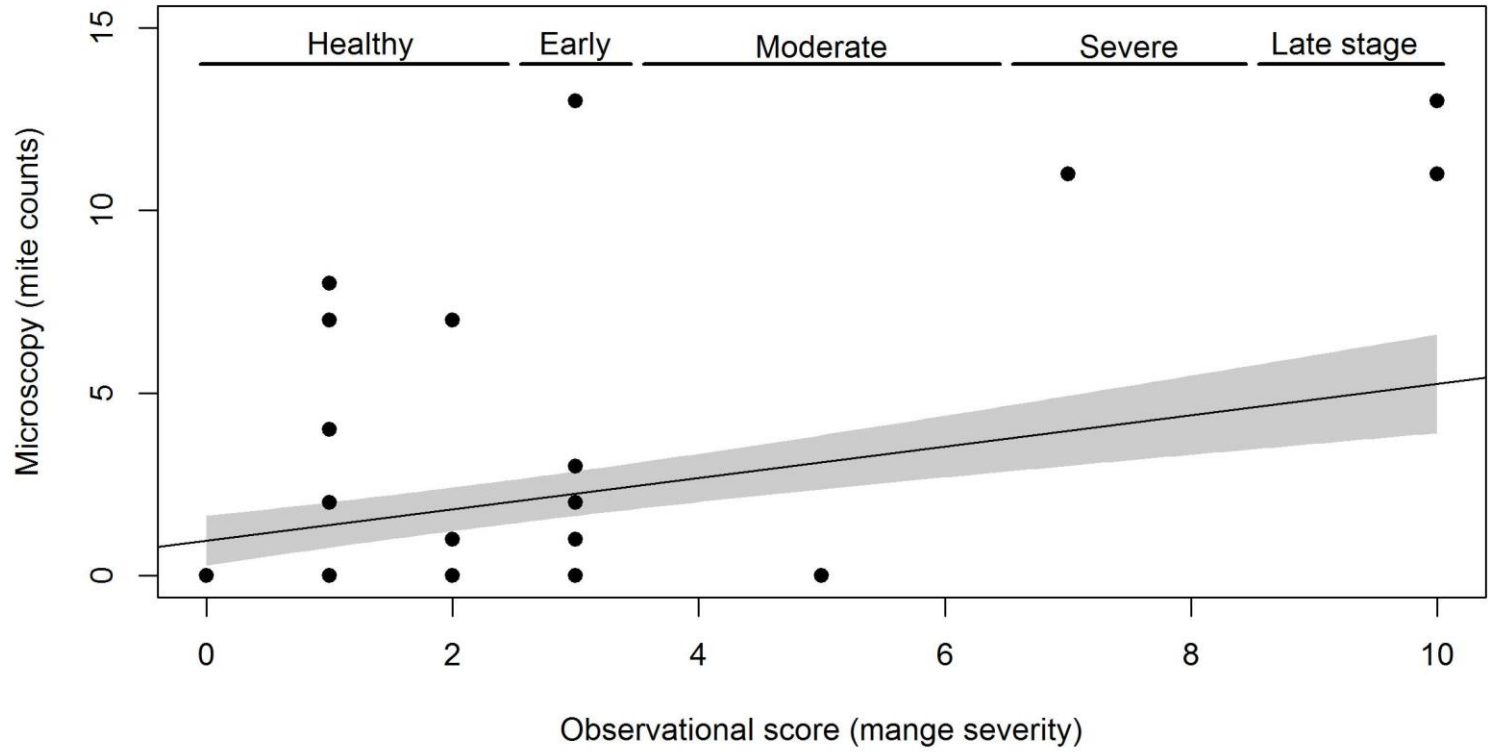

Figure 5.3: Mange score and microscopy association. 40 skin scrapings from 23 wombats, with a correlation between mange score and mite counts. Line of best fit with 95% CIs included for illustrative purposes.

of *S. scabiei* infestation, relative to microscopy, was 57.14% (95% CI: 28.86% – 82.34%), and specificity was 88.46% (95% CI: 69.85% – 97.55%) (Table 5.2). Cohen's Kappa indicated agreement between the two diagnostic tests to be moderate (47.98%, 95% CI: 19.15% – 76.80%).

5.4.2 PCR on skin scrapings identifies more *S. scabiei* infested individuals than microscopy

We applied a novel cox1-specific conventional PCR to the total of 40 skin scrapings collected as a part of this investigation and whose mite load was simultaneously assessed by microscopy. In comparison to microscopy, PCR of skin scraping was 100% (95% CI: 76.84% – 100%) sensitive, with a specificity of 84.62% (95% CI: 65.13% – 95.64%). PCR negative skin scrapings were always negative by microscopy, but 4/26 samples that were negative by microscopy proved to be positive by PCR (Table 5.2), causing the lower specificity in this instance. The agreement between microscopy and PCR of skin scrapings was substantial (Cohan's Kappa 79.38%, 95% CI: 60.62% – 98.14%). To validate that the four PCR positive and microscopy negative samples were not the result of nonspecific amplification, sequencing was performed and confirmed *S. scabiei* PCR positivity. Haplotype sequence can be found in Fig.5.2 labelled cox1 PCR sequence.

While no microscopy positive samples were negative by mite PCR, we were nevertheless concerned about an absence of amplifiable nucleic acid that could cause a 'false-negative' confounder in the PCR assay. As such, we separately performed an additional beta actin qPCR to test for the amplification of wombat genomic DNA in cox1 PCR negative skin scrapings. Of the latter group of 21 negative cox1 skin scrapings, a wombat beta actin 111 bp PCR product could be amplified for 19/21 (90.47%). The two samples that failed this control amplification (W009 and W017) produced melt curves at 80 °C, but the relative fluorescence units (RFU)

Table 5.2: Concordance between microscopy and alternative diagnostic tests for *S. scabiei* detection.

Alternative Test	Microscopy		Total (%)	Kappa (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
	Detected	Not Detected				
Mange Score						
Mangy	8	3	*11 (27.5)*	47.98% (19.15 - 76.80)	57.14% (28.86 - 82.34)	88.46% (69.85 - 97.55)
Healthy	6	23	*29 (72.5)*			
Total (%)	*14 (35)*	*26 (65)*	*40*			
Skin Scraping PCR						
Positive	14	4	*18 (35)*	79.38% (60.62 - 98.14)	100% (76.84 - 100)	84.62% (65.13 - 95.64)
Negative	0	22	*21 (65)*			
Total (%)	*14 (45)*	*26 (55)*	*40*			
Swab PCR						
Positive	6	1	*7 (36.84)*	67.05% (33.13 - 100.98)	75.00% (35.91 - 96.81)	90.91% (58.72 - 99.77)
Negative	2	10	*12 (63.16)*			
Total (%)	*8 (42.11)*	*11 (57.89)*	*19*			

were below detection levels, indicating that collection of host cells was limited but still successful.

5.4.3 PCR on swab samples may not be as effective a method for *S. scabiei* detection

We investigated the use of PCR testing of swab samples for identification of *S. scabiei* on 19/23 wombats, that were both swabbed and skin scraped at an adjacent site. Sensitivity of swab PCRs, relative to microscopy on skin scrapings was 75.00% (95% CI: 35.91% – 96.81%) and specificity 90.91% (95% CI: 58.72% – 99.77%) (Table 5.2). Cohan's Kappa indicate a substantial agreement between the two diagnostic methods at 67.05% (95% CI: 33.13% – 100.98%). While the overall congruence was high, wombat beta-actin PCR testing of cox1 PCR negative swabs only detected 2/12 (16.67%) to be positive, indicating that collection of host cells was scarce. The single false positive sample (W004) was confirmed to be positive by PCR sequencing.

5.4.4 Overall consensus identifies skin scraping PCRs are most consistent with microscopy

Nineteen wombats in the current investigation were assessed by all four sampling/diagnostic methods simultaneously (mange score, microscopic examination of skin scraping, cox1 PCR of skin scraping and cox1 PCR of swab sample) at the same anatomical site. Diagnostic test results for each of these is described in Table 5.3. Result congruence could be observed in 14/19 wombats (73.68%), comprising of 4 positive for *S. scabiei* and 10 negative. 100% agreement could be observed between microscopy and skin scraping PCR between the 19 animals (N=8 positive, 11 negative). 84.21% congruence could be observed between microscopy/skin scraping PCR and swab PCR, with one animal (W004) being positive via swab PCR and swab PCR sequencing (and mange score of 5), but negative for microscopy and skin scraping PCR, and two wombats (W021 and W027) being negative by swab PCR but

Table 5.3: Assessment of all four diagnosis methods for 19 wombats shows a general consensus between tests.

Wombat ID	Microscopy mite count	Body Segment Mange Score	Skin Scraping PCR	Swab PCR
DW02	11	10	Positive	Positive
W002	0	1	Negative	Negative
W003	13	10	Positive	Positive
W004	0	5	Negative	Positive
W005	13	3	Positive	Positive
W006	2	3	Positive	Positive
W008	8	1	Positive	Positive
W009	0	0	Negative	Negative
W010	0	0	Negative	Negative
W013	0	0	Negative	Negative
W014	0	0	Negative	Negative
W016	1	2	Positive	Positive
W018	0	0	Negative	Negative
W019	0	0	Negative	Negative
W020	0	0	Negative	Negative
W021	7	2	Positive	Negative
W024	0	0	Negative	Negative
W025	0	2	Negative	Negative
W027	3	3	Positive	Negative

19 wombats had a mange score, microscopy mite count, skin scraping PCR and swab PCR results from the same body region (flank). Mange scores between 0 – 2 are classified as healthy and mange scores >3 are classified as mangy. The remaining four wombats (W015, W017, W022 and W023) were not included in this table as skin scrapings and swabs were not collected from these individuals flank.

positive for the remaining methods (Table 5.3). These results support the above findings, particularly in regards to higher detection success of overtly healthy individuals and severe mange infestation.

5.5 Discussion

Despite the significance of *S. scabiei* to humans and other animals globally, clinical diagnosis of infestation (particularly false-negatives) remains a major challenge. Taking advantage of a unique sample set from bare-nosed wombats, we evaluated several techniques commonly used or recently proposed for identifying *S. scabiei* in hosts (observational scoring, PCR on skin scrapings, and PCR on skin swabs) and compared these to the standard diagnostic of microscopy on skin scrapings. Our results show that there is a positive correlation between the presence of mites detectable by skin scrapings and observational scores. However, this technique is conservative, as the sensitivity of this approach is lower in animals with low numbers of mites. We found that *S. scabiei* specific PCR on skin scrapings had enhanced sensitivity relative to microscopic detection of the mites, and that non-invasive sampling methods via PCR on skin swab samples can also be used, but with lower sensitivity to the aforementioned methods. Overall, our results identify PCR on skin scrapings as an advancement on traditional microscopy, and highlight the more conservative nature (greater false-negatives) of other techniques tested.

Field observational diagnosis for sarcoptic mange and its severity is simple and effective for advanced mange, as indicated by alopecia and with crusted scabies due to the relatively unique signs of disease [14, 156]. This method is particularly valuable as a tool for field assessment of mange, as it means animals do not need to be captured and stressed. While this methodology has been applied across several wild mammal species e.g., PC Cross, ES Almberg, CG Haase, PJ Hudson, SK Maloney, MC Metz, AJ Munn, P Nugent, O Putzeys and DR Stahler [157] and

AM Martin, CP Burridge, J Ingram, TA Fraser and S Carver [38], the current study is the first to quantify the diagnostic relevance of observational scoring, relative to microscopic examination of skin scrapings. This analysis revealed a positive correlation between mange severity and mite load. As anticipated, the sensitivity of observational scoring was low, relative to microscopy, owing to non-detection of early stage infestation, when mite numbers are low. In a recent review, LG Arlian and MS Morgan [14] noted that *S. scabiei* mites appear to have immune suppressing mechanisms during early stages of infestation. Even when symptoms do appear, it may be some time (weeks) before observational diagnoses can be reliably made. Accordingly, scoring of mange in field settings should always be considered an underestimate of true prevalence (indeed most studies do), which our study suggests may be by >40% underestimation.

Our study adds to a growing body of research suggesting PCR on skin scrapings is a valuable tool for improved detection of *S. scabiei* [151, 158-160]. We found PCR detection of mites when they were present was largely congruent with microscopy, and detected mites in cases where microscopy did not. Interestingly, our results also provide some preliminary insight on how a single skin scraping (1–2cm2 area) may be insufficient for an accurate diagnosis of low level mite infestation (one wombat, W027, receiving three skin scrapings from the same body segment were positive by microscopy 2/3 times and all three times by PCR). These results suggest that *S. scabiei* PCR assays on skin scrapings may be a more sensitive technique for detection of mites than microscopy, particularly when hosts have low levels of mite infestation. It has been suggested that mite excreta and mite cellular debris are deposited into the stratum corneum to which PCR can detect and human examination will miss [151]. On the basis of these results, we suggest that, wherever possible, PCR detection of *S. scabiei* should be considered in the suite of diagnostic tools available for this disease. The obvious limitation of this latter approach is the technical and logistical requirements for a nuclear acid amplification

test which may not be available in the field or in a typical veterinary clinic. In the absence of such a test, there may be opportunities in the future for the development of a point-of-care nucleic acid amplification tests for rapid, specific and low cost detection of *S. scabiei* DNA with simple benchtop equipment or even in field settings [161]. This study also offered the opportunity to evaluate the use of swabs as a new non-invasive (not skin-scraping) method of sampling *S. scabiei*. A previous human study found that skin swabs from multiple areas on the body of a single crusted scabies patient were 100% successful in detecting mite DNA by PCR, but any comparison to microscopy and scraping PCR over the treatment period was lacking [151]. In the current study we were successful in detecting mite DNA in 75% of swab samples that were also microscopically positive. Our results suggest an increased risk of false-negatives associated with PCR on swabs, relative to microscopy and also PCR on skin scrapings. This is evident by beta actin assays only confirming 16.67% of the negative cox1 samples to be positive for wombat beta actin, suggesting that the uptake of host skin containing any potential mites was insufficient. Future directions surrounding diagnostic swabs should investigate cyto-brushes as they are designed to collect a large number of host cells and minimise false-negatives.

A variety of research calls for new or additional diagnostic tools for *S. scabiei* to be coupled with the standard technique of microscopy [19, 151, 162, 163]. Here, we tested a selection of putatively useful diagnostic approaches for *S. scabiei*. To the best of our knowledge, this is the first study to simultaneously assess this combination of diagnostic methods. Using our modest sample size, we identified that PCR on skin scrapings provided a higher level of detection than microscopy, and is superior to swabs for mite detection. It is suggested, however, that any PCR positive result which is not supported by microscopic identification should be additionally confirmed by amplicon sequencing. Observational scoring of *S. scabiei* infestation was also useful, but highly conservative at low levels of infestation. In spite of these methods having a

high level of association, we provide evidence to suggest that a single skin scraping may be insufficient for low mite infestation as multiple skin scrapings from the same body region can produce conflicting results. The results from this unique study are not limited to wombats specifically, but can be used as fundamentals for future diagnostic research for other *S. scabiei* host species, including humans.

CHAPTER 6

A *SARCOPTES SCABIEI* SPECIFIC ISOTHERMAL AMPLIFICATION ASSAY FOR DETECTION OF THIS IMPORTANT ECTOPARASITE OF WOMBATS AND OTHER ANIMALS

Tamieka A. Fraser[a,b], Scott Carver[a], Alynn M. Martin[a], Kate Mounsey[b,c], Adam Polkinghorne[b], Martina Jelocnik[b*]

[a] Department of Biological Sciences, University of Tasmania, Sandy Bay, Tasmania, Australia

[b] USC Animal Research Centre, Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, Sippy Downs, Queensland, Australia

[c] School of Health and Sport Sciences, University of the Sunshine Coast, Sippy Downs, Queensland, Australia

This paper was published in PeerJ, 27th July 2018, DOI 10.7717/peerj.5291.

Statement of joint authorship

TAF, MJ, SC and AP conceptualised the content of this manuscript. Samples were collected from TAF, AMM and KM. Experiments were performed by TAF and MJ. TAF conducted analysis and wrote this manuscript with drafting contributions and approval from remaining authors.

6.1 Abstract

Background: The globally distributed epidermal ectoparasite, *Sarcoptes scabiei*, is a serious health and welfare burden to at-risk human and animal populations. Rapid and sensitive detection of *S. scabiei* infestation is critical for intervention strategies. While direct microscopy of skin scrapings is a widely utilised diagnostic method, it has low sensitivity. PCR, alternatively, has been shown to readily detect mite DNA even in microscopy-negative skin scrapings. However, a limitation to the latter method is the requirements for specialised equipment and reagents. Such resources may not be readily available in regional or remote clinical settings and are an important consideration in diagnosis of this parasitic disease.

Methodology: A Loop Mediated Isothermal Amplification (LAMP) assay targeting the ITS-2 gene for *S. scabiei* was developed and evaluated on clinical samples from various hosts, previously screened with conventional S. scabies-specific PCR. Species specificity of the newly developed LAMP assay was tested against a range of DNA samples from other arthropods. The LAMP assays were performed on a real-time fluorometer as well as thermal cycler to evaluate an end-point of detection. Using skin scrapings, a rapid sample processing method was assessed to eliminate extensive processing times involved with DNA extractions prior to diagnostic assays, including LAMP.

Results: The *S. scabiei* LAMP assay was demonstrated to be species-specific and able to detect DNA extracted from a single mite within a skin scraping in under 30 minutes. Application of this assay to DNA extracts from skin scrapings taken from a range of hosts revealed 92.3% congruence (with 92.50% specificity and 100% sensitivity) to the conventional PCR detection of *S. scabiei*. Preliminary results have indicated that diagnostic outcome from rapidly processed dry skin scrapings using our newly developed LAMP is possible in approximately 40 minutes.

Discussion. We have developed a novel, rapid and robust molecular assay for detecting *S. scabiei* infesting humans and animals. Based on these findings, we anticipate that this assay will serve an important role as an ancillary diagnostic tool at the point-of-care, complementing existing diagnostic protocols for *S. scabiei*.

Keywords: LAMP, Diagnostics, Sarcoptic Mange, Skin Scraping, PCR, One Health, Australian Wildlife, *Sarcoptes scabiei*

6.2 Introduction

Sarcoptes scabiei is an ectoparasite that resides in the epidermal layer of its hosts causing a range of clinical signs of disease including pruritis, dermal inflammation, hyperkeratosis and alopecia, which may lead to bacterial sepsis [23]. *S. scabiei* is listed among the top 50 most prevalent diseases in humans with over 100 million people globally predicted to be infested [164, 165]. Beyond its role in human disease, a wide range of domestic animals, wild canids, and other wildlife suffer extensively from sarcoptic mange, and transmission to at-risk animal populations can result in population declines and localised extinctions [112, 166-169]. With the endemic infestation of humans in tropical and subtropical areas, the large variety of animal species infested and the knowledge that *S. scabiei* is the same mite infesting all, pathogen dispersal and spill-over has been suggested to be the causative consequence of global infestations [19, 128].

As with many infectious diseases, the successful treatment of affected individuals and the application of appropriate disease management strategies relies on rapid and accurate detection of the infectious agent. Diagnosis of scabies (also classified as mange in animals) is typically made by assessment of clinical features alone [19, 170]. When atypical appearances are presented, however, the diagnosis can be challenging as other skin conditions can mimic clinical signs of scabies [170]. A skin scraping of the affected area provides a more definitive

diagnosis as mites, mite eggs and faecal pellets can be identified via microscopy due to their distinct morphology [36]. Although the diagnosis is more specific using microscopy, detection of early mange has been shown to have limited sensitivity, primarily due to the difficulties in sampling and visualising mites when the mite burden is low [19, 34, 171]. Recent studies have shown that diagnosis of *S. scabiei* by clinical features and microscopy are unreliable methods for early stage infestations [151, 171].

Besides microscopy, alternative diagnostic methods for *S. scabiei* have been evaluated with varying sensitivity and specificity. Several studies have attempted to use serological techniques (i.e., ELISAs) as a more targeted diagnostic method [48, 51, 52, 107, 172]. However, as reviewed by Arlian & Morgan [14], significant limitations for this method exist including the time taken for the host to develop *S. scabiei*-specific antibodies and cross-reactivity between *S. scabiei* antigens and those from other mites. Molecular techniques using nucleic acid amplification tests (NAATs) as a diagnostic tool for *S. scabiei* are relatively new but show promising results [151, 171]. Two studies, analysing samples collected from humans [151] and animals [171], have demonstrated that PCRs have a higher sensitivity and specificity than microscopy, revealing high rates of false negative samples previously screened by microscopy. However, NAATs are not well adapted for clinical settings, particularly for diseases like scabies which are common in remote or resource- limited communities and/or in field settings with limited access to diagnostic laboratories and necessary equipment [19]. Recent advances in this field have utilised hand held devices resulting in promising outcomes for detecting infectious diseases quickly. This includes the Biomeme Inc. portable PCR machine with thermocycler and fluorometer which can dock into an iPhone resulting in rapid results and the AmplifyRP® portable florescence reader [173, 174].

Loop mediated isothermal amplification (LAMP) is one of the expanding range of NAAT techniques that is showing capacity at the Point of Care (POC). LAMP assays are low cost, rapid and can be used with simple 'bench-top' equipment. Visual result interpretation with the use of different DNA binding dyes in these assays further support LAMP use at the POC. There have been multiple successful LAMP assays developed for other human and veterinary parasites including *Plasmodium* spp. [175], *Toxoplasma gondii* [176] and *Leishmania* spp. [177], and bacteria including *Chlamydia* spp. [178], *Mycoplasma pneumoniae* [179] and *Streptococcus agalactiae* [180]. To overcome on some limitations associated with LAMP assays, including misleading of results using turbidity techniques and cross contamination as a result of opening tubes, the use of a fluorometer and a signature melt for amplicon characteristics can account for these limitations.

This study aimed to develop a LAMP assay for the detection of *S. scabiei* in animals and assess its reliability against PCR and demonstrate its potential as a POC test. We have utilised a unique sample set of skin scrapings taken from a range of hosts and tested extracted DNA from those with the newly developed *S. scabiei* specific LAMP assay. The LAMP assay was evaluated against microscopy and a recently described conventional *S. scabiei*-specific PCR assay. In an attempt to reduce sample processing time, we also optimised a rapid DNA extraction method on a small subset of skin scrapings, further highlighting the potential for this assay to be deployed at the POC.

6.3 Methods and Materials

6.3.1 LAMP assay design

The *S. scabiei* internal transcribed spacer 2 (ITS-2) gene is a highly conserved gene and was used as the LAMP target in this study. A ClustalW alignment of 87 ITS-2 sequences (represented as haplotypes) from *S. scabiei* mites from humans and a variety of animals

across Australia, Europe, North America and Asia available in GenBank was obtained to identify polymorphisms in this gene (Fig. 6.1, Table 6.1). In addition, we have included ITS-2 sequences from other mite species, including the house dust mite (*Dermatophagoides farinae*), the chorioptic mange mite (*Chorioptes* sp), the notoedric mange mite (*Notoedres cati*), the psoroptic mange mite (*Psoroptes* sp) and ticks (*Ixodes* sp). This 450 bp fragment was also subjected to a discontiguous megablast search in Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi#) [181] to evaluate *S. scabiei* sequence specificity. Primer explorer V5 (Eiken Chemical Co., Tokyo, Japan) was used for primer design and yielded five sets of primers consisting of two outer (F3 and B3) and two inner (FIP and BIP) primers. These sets were analysed and three were excluded due to sequence overlap and primer parameters. The remaining two sets were selected for further testing with both sets analysed in silico with BLAST [181] and the OligoAnalyzer 3.1 online tool (https://sg.idtdna.com/calc/analyzer) [182] to assess primers for DNA base mismatches, hairpins and annealing temperature. Loop primers were additionally designed manually to increase sensitivity and accelerate the reaction time (Fig. 6.2, Table 6.2).

6.3.2 *S. scabiei* LAMP assay validation

Each LAMP reaction consisted of a 15 µL Isothermal Master Mix ISO001 (Optigene, Horsham, UK), 5 µL primer mix (at 0.2 µMF3 and B3, 0.8 µMFIP and BIP, and 0.4 µM LF and LB) and 5 µL of DNA template. Initial testing and validation of the two primer sets was performed on *S. scabiei* DNA from a single mite and a pool of three mites at 65°C for 30 min using a heating block, with results visualised on an ethidium bromide agarose gel under UV light. During this development step, the second primer set was excluded due to high primer dimerization (not shown), and LAMP primers described in Fig. 6.2 and Table 6.2 were used henceforth. Confirmation of the LAMP target sequence was completed by sequencing the

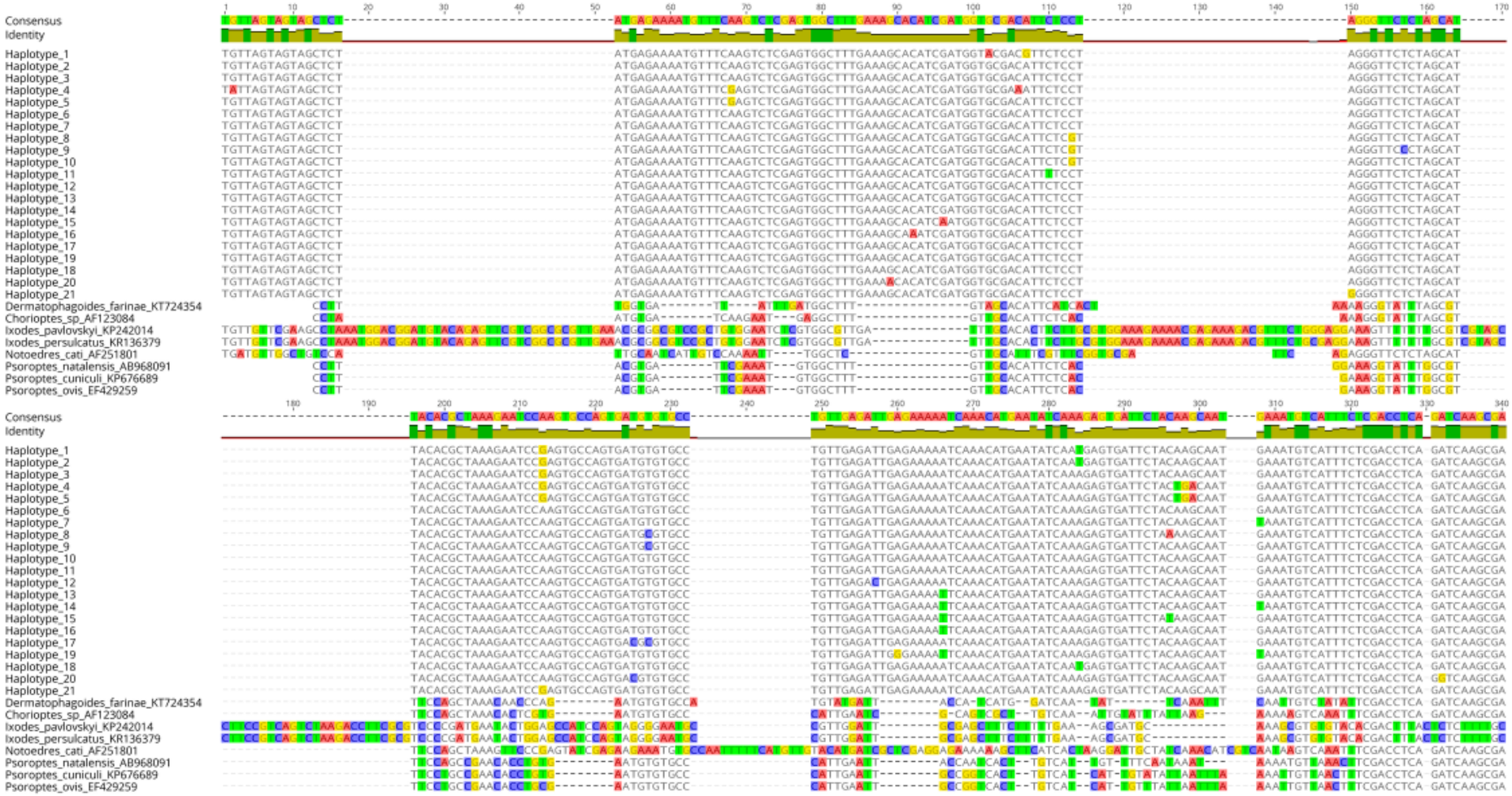

Figure 6.1 Alignment of the available *S. scabiei* and other arthropod ITS-2 gene sequences from GenBank

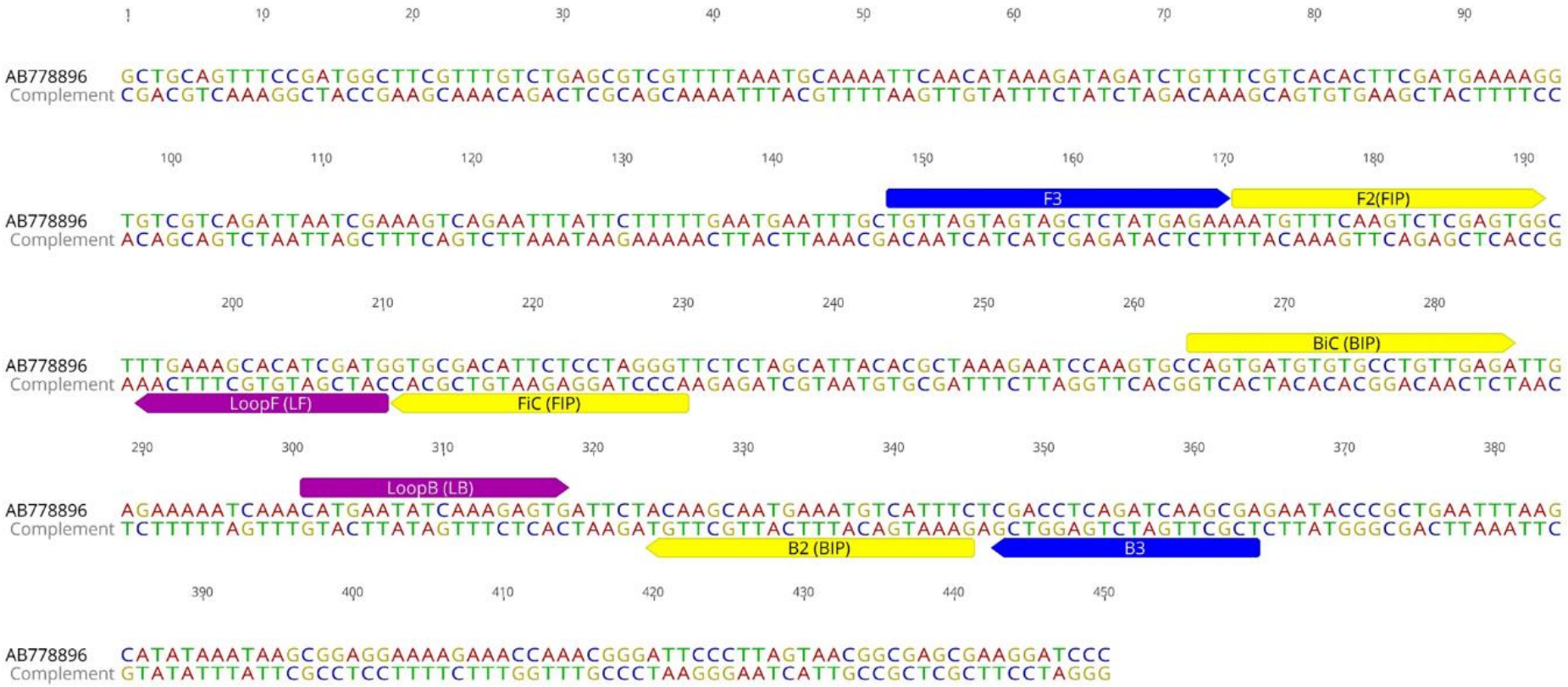

Figure 6.2: *S. scabiei* LAMP primer sequences. Two outer (F3 and B3), two inner (FIP and BIP) and two looping (LF and LB) primers for S. scabiei specific LAMP assay outlined on the ITS-2 sequence (Genbank accession number AB778896).

Table 6.1. Accession numbers of the 87 ITS-2 sequences obtained from GenBank for alignment

Haplotype number	Number of sequences	Genbank Accession number
1	1	AM980728
2	6	AB820967, AM980803, AM980806, AM980812, AF129151, KJ739610
3	3	AF387705, AB535737, AF387719
4	1	AM980775
5	1	AM980818
6	35	AB778900, AB778901, AB778898, AB778899, AF387723, AF387722, AF387724, AF387725, AF387703, AM980686, AF129145, AF129149, AF129148, AF129150, AF129147, AF129142, AF129143, AM980731, AB820972, AB778917, AF129164, AF129163, AF129152, AF387712, AF129160, AF129154, AF129153, AB778896, AB778897, AB778895, AB778905, AB778912, AF387704, AF129157, AM980750
7	2	AF129161, KJ739612
8	1	AM980679
9	1	AF129146
10	4	AM980689, AF129156, AF129155, AF129158
11	1	AM980758
12	1	AM980799
13	21	AB778902, AB778903, AF387730, AB778904, AB778906, AB778907, AB778908, AB778909, AB778910, AB778911, AB778913, AB778914, AB778915, AM980757, AB778916, AB778918, AM980681, AB778919, AF129159, AF387729, AF387727
14	1	AM980808
15	1	AM980773
16	2	AM980721, AM980751
17	1	AM980770
18	1	AF129144
19	1	KJ739613
20	1	KJ739611
21	1	KJ739615

Table 6.2: LAMP primers used in this study.

Name	Sequence 5'-3'	Position	Length
F3	TGTTAGTAGTAGCTCTATGAGAA	148-170	23
B3	TCGCTTGATCTGAGGTCG	364-347	18
FIP (FiC + F2)	ACCCTAGGAGAATGTCGCACAATGTTTC AAGTCTCGAGTGG		41
BIP (BiC + B2)	CAGTGATGTGTGCCTGTTGAGAGAAATG ACATTTCATTGCTTGT		44
Loop F	CATCGATGTGCTTTCAA	210-194	17
Loop B	CATGAATATCAAAGAGTG	301-318	18
F2	AATGTTTCAAGTCTCGAGTGG	171-191	21
FiC	ACCCTAGGAGAATGTCGCAC	230-211	20
B2	CAGTGATGTGTGCCTGTTGAGA	345-324	22
BiC	GAAATGACATTTCATTGCTTGT	264-285	22

amplification product generated with outer F3 and B3 primers, with the resulting sequence deposited in Genbank under accession number MH379093

After initial optimisation, samples were tested using the Genie III real-time fluorometer (Optigene, Horsham, UK), and reactions were run at 65°C for 30 min, followed by annealing at 98°C to 80°C at a rate of 0.05°C /s to generate the signature melt profile (curve) of the amplified product. A negative control consisting of water as template was included in each run.

LAMP gene target specificity was evaluated using other arthropod DNA (*Pediculus humanus, Leptotrombidium pallidum, Periplaneta australasiae, Bovicola ovis, Bovicola bovis, Solonopotes capillatus, Ixodes holocyclus, Ixodes tasmani*), and skin scrapings negative for *S. scabiei* (as previously determined by PCR and microscopy [171]).

6.3.3 Clinical samples used in this study

The evaluation of the *S. scabiei* LAMP assay was performed on (i) DNA extracts from 40 skin scrapings collected from 23 wombats (Vombatus ursinus) as previously described [171] and, (ii) 24 DNA extracts from individual skin scrapings collected from five domestic dogs (Canis lupus familiaris), eight wombats, two koalas (Phascolarctos cinereus), two wallabies (Macropodidae sp.), and seven known healthy wombats, stored in 80% ethanol at −80°C (Table 6.3). The DNA extraction procedure was performed as previously described using QiaAMP DNA Mini kit (Qiagen, Valencia, CA, USA) [171]. The collection and use of these samples was approved by the Animal Research Committee at the University of the Sunshine Coast (approval AN/S/16/43, and AN/E/17/17), the Animal Research Committee at the University of Tasmania (approval A0014670) and state permits from Office of Environment & Heritage NSW National Parks & wildlife Service (SL101719), Department of Primary Industries, Park, Water and Environment for Tasmania (approval FA15121) and The

Table 6.3: Results for microscopy mite count, PCR and LAMP for all samples used in this study. Each sample has a corresponding host, microscopy mite count, skin scraping PCR and LAMP result. In some instances, multiple skin scrapings were taken from an individual, reflected by the sample name_body location. LF: left flank, RF: right flank, N: neck, H: head, RA: right forearm, RL: right hind leg, B: back, LL: left hind leg. If multiple skin scrapings were taken from the same body location this is described by the number next to name_bodylocation.

Host	Sample Name	Mite Count	Skin Scraping 374PCR	LAMP	Time to amplify (min)	Melt (°C)
Wombat	DW02_RL1	11	Positive	Positive	10.15	85.34
Wombat	DW02_RL2	NA	Positive	Positive	11.15	85.54
Wombat	W002_LF	0	Negative	Negative		
Wombat	W002_RF	2	Positive	Positive	15.30	85.51
Wombat	W003_RF	13	Positive	Positive	11.00	85.65
Wombat	W004_N	2	Positive	Positive	12.30	85.32
Wombat	W004_RF	0	Negative	Positive	14.30	85.36
Wombat	W005_RF	13	Positive	Positive	11.45	85.47
Wombat	W006_H	0	Positive	Positive	20.30	85.43
Wombat	W006_RF	2	Positive	Positive	11.45	85.48
Wombat	W008_RF	8	Positive	Positive	12.00	85.43
Wombat	W009_RF	0	Negative	Negative		
Wombat	W010_RF	0	Negative	Negative		
Wombat	W013_LF	0	Negative	Negative		
Wombat	W013_RA	0	Negative	Negative		
Wombat	W014_LF	0	Negative	Negative		
Wombat	W014_RL	0	Negative	Negative		
Wombat	W015_RA	0	Positive	Positive	13.15	85.18
Wombat	W016_LF	1	Positive	Positive	11.45	85.45
Wombat	W016_N	7	Positive	Positive	11.00	85.33
Wombat	W016_RL	4	Positive	Positive	13.00	85.20
Wombat	W017_B	0	Negative	Positive	No time recorded	85.78
Wombat	W017_RL	0	Negative	Negative		
Wombat	W018_RF	0	Negative	Negative		
Wombat	W019_RA	0	Negative	Negative		
Wombat	W019_RF	0	Negative	Negative		
Wombat	W020_RF	0	Negative	Negative		
Wombat	W021_RL	11	Positive	Positive	12.00	85.28
Wombat	W021_RF	7	Positive	Positive	10.45	85.48
Wombat	W022_LL	0	Negative	Negative		
Wombat	W023_LL1	0	Negative	Negative		

Wombat	W023_LL2	0	Negative	Negative		
Wombat	W023_LL3	0	Negative	Negative		
Wombat	W024_RF1	0	Negative	Negative		
Wombat	W024_RF2	0	Positive	Positive	15.00	85.80
Wombat	W025_LF1	0	Negative	Negative		
Wombat	W025_LF2	0	Negative	Negative		
Wombat	W025_LF3	0	Negative	Negative		
Wombat	W027_RF1	3	Positive	Positive	12.30	85.29
Wombat	W027_RF2	1	Positive	Positive	14.00	85.33
Wombat	W027_RF3	0	Positive	Positive	20.00	85.08
Dog	NT1	NA	Positive	Positive	12.00	85.42
Dog	NT2	NA	Positive	Positive	15.00	85.62
Dog	NT3	NA	Negative	Negative		
Dog	NT4	NA	Positive	Positive	No time recorded	85.54
Dog	NT5	NA	Positive	Positive	14.45	85.68
Koala	KSA1	NA	Positive	Positive	11.00	85.40
Wombat	WV1	NA	Negative	Negative		
Wombat	WV2	NA	Negative	Negative		
Wombat	WV3	NA	Negative	Positive	No time recorded	85.08
Wombat	WV4	NA	Negative	Negative		
Wombat	WV5	NA	Negative	Negative		
Wombat	WV6	NA	Negative	Negative		
Wombat	WV7	NA	Negative	Negative		
Wallaby	WaV1	NA	Negative	Negative		
Wallaby	WaV2	NA	Negative	Negative		
Koala	KV1	NA	Negative	Negative		
Wombat (negative)	WT1	0	Negative	Negative		
Wombat (negative)	WT2	0	Negative	Negative		
Wombat (negative)	WT3	0	Negative	Negative		
Wombat (negative)	WT4	0	Negative	Negative		
Wombat (negative)	WT5	0	Negative	Negative		
Wombat (negative)	WT6	0	Negative	Negative		
Wombat (negative)	WT7	0	Negative	Negative		
S. scabiei	Ss2	1	Positive	Positive	20.00	85.26
S. scabiei	Ss3	3	Positive	Positive	11.30	85.39

NA: not applicable

Department of Environment, Land, Water and Planning for Victoria (10007943). All methods were carried out in accordance with the 2013 Australian National Health and Medical Research Council 'Australian code for the care and use of animals for scientific purposes'. Aforementioned samples were also screened by conventional PCR targeting a 374 bp fragment of the *S. scabiei* cox1 gene, having a respective sensitivity and specificity of 100% and 84.62% in concordance to microscopy, as previously described [171]. PCR positivity for cox1 was determined by visualisation of the 374bp fragment following agarose gel electrophoresis under UV light.

In order to confirm negative samples and to test for isothermal amplification inhibition, a subset of six negative samples were spiked with 10 µL mite only DNA and tested again by LAMP.

6.3.4 Evaluation of rapid skin scraping DNA extraction

In order to assess the use of LAMP at the POC, eleven wombat skin scrapings, with mite counts previously assessed by microscopy, were submerged with 0.3M Potassium Hydroxide (KOH), pH 13, and heated at 95°C for 10 min in order to lyse the tissue and release DNA from the cells. After vortexing, 5 µL of the tissue suspension was used as a template in each reaction, also consisting of 15 µL of Lyse'n'Lamp master mix (Optigene, Horsham,UK)and 5µL primer mix as described above. TheLAMPreaction was performed in the Genie III fluorometer using the same cycling conditions as described above. Negative controls of water only and an aliquot of 0.3M KOH only were included in the assays. The same samples were also tested with the cox 1 PCR after performing DNA extractions, as described above, on the KOH skin suspensions.

6.3.5 Statistical analysis

The performance of the LAMP assay compared to the reference PCR assay conducted on the same samples was estimated by calculating Kappa values, overall agreement, sensitivity and specificity. Direct comparisons were conducted using EpiTools online (http://epitools.ausvet.com.au) [183]. Kappa values are interpreted as follows: values ≤0 as indicating no agreement, 0.01–0.20 as none to slight, 0.21–0.40 as fair, 0.41–0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1.00 as almost perfect agreement.

6.4 Results

6.4.1 *S. scabiei* LAMP assay development and validation

The LAMP primers were predicted to amplify a 217 bp fragment of the ITS-2 gene. The alignment of the available *S. scabiei* ITS-2 gene sequences (n=87) revealed 96.5%–100% sequence identity (Fig. 6.3). In silico analysis of ITS-2 sequences obtained from *Dermatophagoides farinae* (KT724354), *Chorioptes* sp. (AF123084), *Ixodes pavlovskyi* (KP242014), *Ixodes persulcatus* (KR136379), *Notoedres cati* (AF251801), *Psoroptes natalensis* (AB968091), *Psoroptes cuniculi* (KP676689) and *Psoroptes ovis* (EF429259) indicated that the *S. scabiei* LAMP primers are likely to be specific, as we identified 101 to 239 nucleotide polymorphisms between our primers and other arthropod sequences (Fig. 6.3).

The *S. scabiei* LAMP assay was initially assessed in house on a thermal block, with the assay run for 30 min at 65°C. A single mite as well as pooled mite DNA extracts were detectable by LAMP, as visualised by the amplicons on the gel. We also tested 10-fold dilutions of a mite and mite-positive skin scraping DNA samples by LAMP on the thermal cycler in two independent runs using the same run conditions (Fig. 6.4). We successfully amplified 10^{-2} single mite DNA dilutions (0.02 ng/µL of DNA) and 10^{-4} skin scraping DNA dilutions (0.008 ng/µL of DNA) (Fig. 6.4).

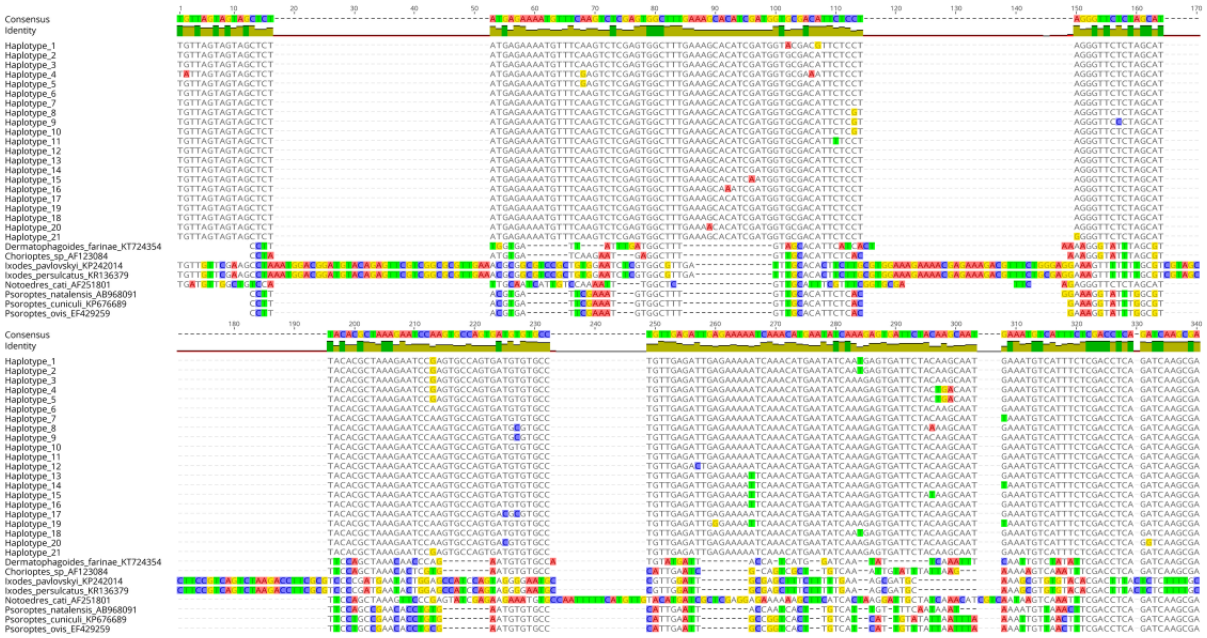

Figure 6.3: Alignment of the available *S. scabiei* and other arthropod ITS-2 gene sequences from GenBank

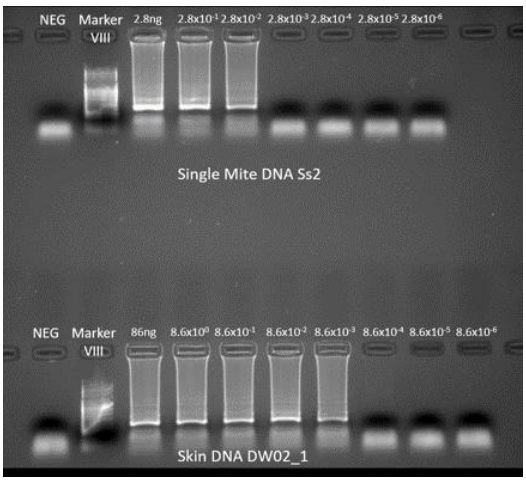

Figure 6.4 S2: End-point detection of S. scabiei LAMP performed on the thermal cycle using 10-fold serial dilutions of single mite and wombat skin DNA. UV visualisation of a single mite (Ss2: 2.8 ng/µL) and a S. scabiei positive wombat skin scraping (DW02_1: 86 ng/µL) LAMP amplicons on an ethidium bromide stained agarose gel. DNA serial dilution LAMP assays were performed on a thermal block for 30 minutes at 65°C. Two negative controls containing water were also included. DNA Molecular Weight Marker VIII (Sigma-Aldrich) was used

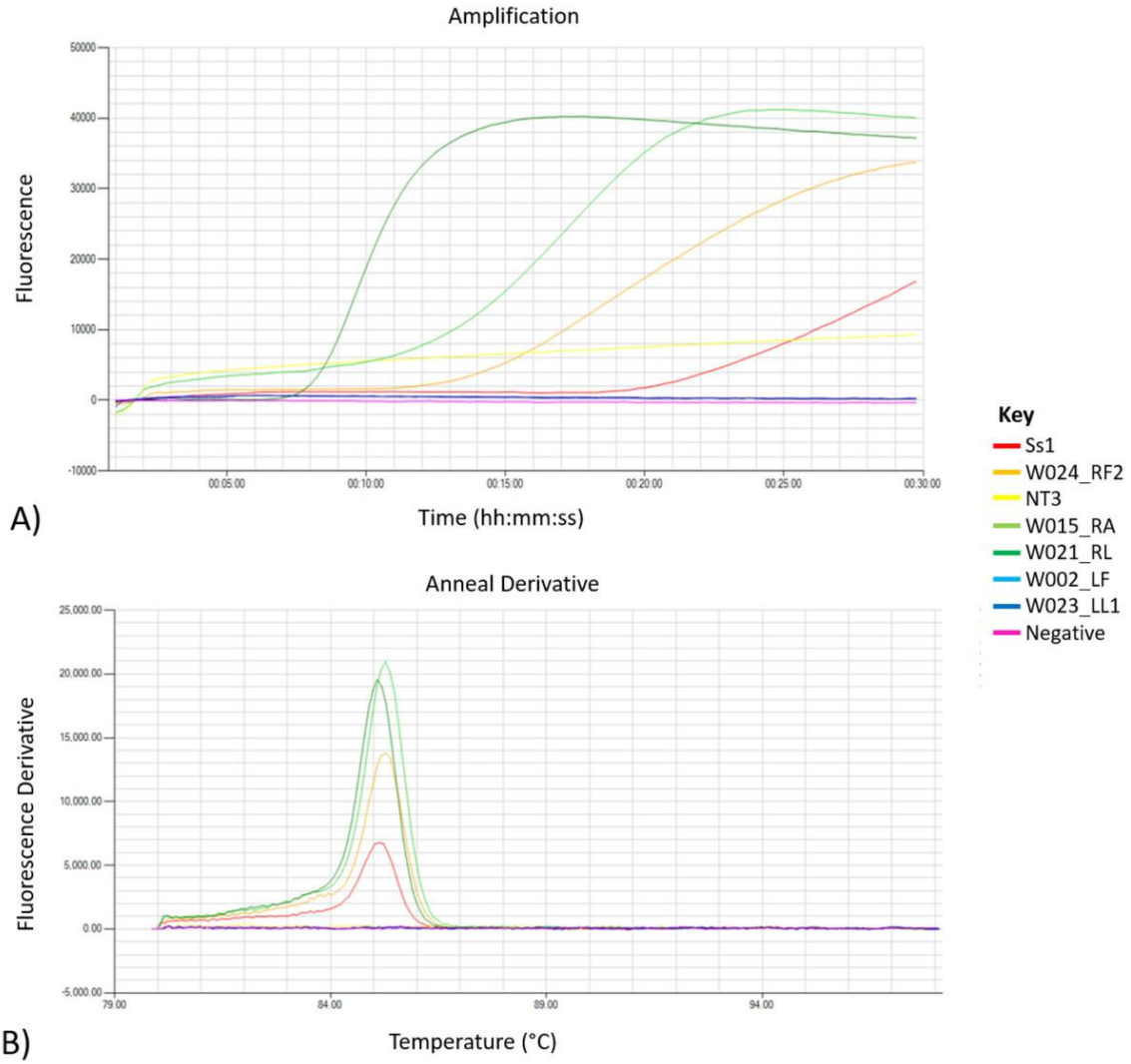

Figure 6.5: Amplification and melt outputs for S. scabiei using specific isothermal amplification. Outputs from the LAMP experimental run; (A) showing amplification and (B) melt outputs using both positive and negative samples in the assay. A water as a template and single mite DNA (Ss2) were included as negative and positive control in the run. Samples with melt at 85 °C are deemed positive.

When the LAMP assay was run in the Genie III fluorometer (Optigene, Horsham, UK) for 30 min at 65°C, DNA extracted from a single mite (Ss2) and a pool of three mites (Ss3) resulted in amplification times of 20.00 and 11.30 min, respectively, with melt temperatures ranging

between 85.26°C to 85.39°C (Fig. 6.5, Table 6.3). Additional validation of species specificity of the *S. scabiei* LAMP assay was performed on a panel of DNA extracts from other arthropods and *S. scabiei* negative skin scrapings on both thermal block and real time fluorometer. None of the other arthropod DNA and previously validated *S. scabiei*-negative skin scraping samples produced LAMP amplification. In comparison to using thermal block for incubation, we found that S. scabies LAMP assays run in the Genie III fluorometer are less laborious and the chance of contamination is decreased as the amplification product is confirmed with its signature melt and is within a closed system.

6.4.2 Validation of the *S. scabiei* LAMP on clinical specimens

A total of 64 clinical samples were tested by the newly developed scabies LAMP, as well as the *S. scabiei* cox1 PCR assay. A high congruence of 95.3% (61/64) was found between the *S. scabiei* LAMP assay and conventional *S. scabiei* PCR assay, previously shown to be more sensitive than microscopy [171], with 24 positive and 37 negative samples in agreement (Table 6.4). Only three samples were positive by *S. scabiei* LAMP but negative by conventional PCR. Overall, LAMP had a sensitivity and specificity of 100% (Clopper–Pearson 95% CI [0.86–1.00]) and 92.50% (Clopper–Pearson 95% CI [0.80–0.98]) respectively when compared to the PCR assay (Table 6.4). Kappa of 0.90 (95% CI [0.84–1.01]) indicated a near perfect agreement between LAMP and the conventional *S. scabiei* cox1 PCR in this study. In order to confirm negative results, six negative samples were ‘‘spiked’’ with mite DNA. All six ‘‘spiked’’ samples produced amplification times of 11.15–16.15 min with melts of 85.28–85.67°C, suggesting that potential inhibitors in the system did not prevent amplification from occurring (Table 6.5).

Table 6.4: Comparison of the S. scabiei LAMP and PCR assays for clinical skin scraping DNA.

PCR	LAMP		Total (%)	Kappa (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
	Positive	Negative				
Positive	24	0	24 (47.44)	0.90 (0.80-1.01)	100% (0.86-1.00)	92.50% (0.80-0.98)
Negative	3	37	40 (52.56)			
Total (%)	27 (51.28)	37 (48.72)	64			

Table 6.5: Spiking results of negative samples to eliminate LAMP inhibition.

Sample	Time to amplify (min)	Melt (°C)
W019_RF	12.3	85.42
WT3	13.45	85.57
WV4	11.15	85.52
NT3	11.15	85.67
WaV1	16.15	85.28
W017_RL	11.15	85.33

Table 6.6: Reproducibility of *S. scabiei* LAMP using skin scraping DNA extracts and mite only DNA.

Samples	Run	Time to amplify (min)	Melt (°C)	Time (Mean + SD)	Melt (Mean + SD)
SS2	1	21.15	85.03		
	2	20	85.26	20.48, 0.60	85.19, 0.14
	3	20.3	85.27		
W024_RF2	1	15	85.3		
	2	12	84.88	13.38, 1.51	85.19, 0.27
	3	13.15	85.38		
NT3	1	Negative			
	2	Negative			
	3	Negative			
W002_RF	1	15.15	85.48		
	2	15	85.28	15.15, 0.15	85.38, 0.10
	3	15.3	85.37		
W002_LF	1	Negative			
	2	Negative			
	3	Negative			
W021_RL	1	9.15	85.14		
	2	9.15	85.09	9.15, 0	85.22, 0.18
	3	9.15	85.42		
W023_LL1	1	Negative			
	2	Negative			
	3	Negative			
WT6	1	Negative			
	2	Negative			
	3	Negative			
WV3	1	No time recorded	84.78		
	2	No time recorded	85.01	No time recorded	85.00, 0.22
	3	No time recorded	85.22		
WV4	1	Negative			
	2	Negative			
	3	Negative			
KSA1	1	11	85.4		
	2	12	85.38	11.43, 0.51	85.40, 0.02
	3	11.3	85.41		
W014_LF	1	Negative			
	2	Negative			
	3	Negative			
W025_LF2	1	Negative			
	2	Negative			
	3	Negative			
W027_RF2	1	14	85.33		
	2	11.15	85.23	12.20, 1.57	85.24, 0.08
	3	11.45	85.17		

6.4.3 Reproducibility

To assess the reproducibility of the LAMP assay, a repeat set of 14 samples were selected and run in triplicate by two operators using blind testing principles (Table 6.6). Assays showed reproducibility of both positive and negative results with small variation between amplification times and melt. Amplification times for each sample varied between 0–3 min and 0.05–0.5 ◦C in melt for each sample.

6.4.4 Rapid specimen processing

We also evaluated the use of an Optigene Lyse'n'Lamp isothermal master mix for rapid DNA extraction prior to *S. scabiei* LAMP. Application of this step to a panel of *S. scabiei* positive (n=7) and negative (n=4) wombat skin scrapings (determined by microscopy), revealed 100% congruence to the microscopy result (Table 6.7) with amplicons generated in positive samples with a time range of 13.2–26.0 min and melts of 84.28–85.20°C. As previously noted by Fraser et al. [171], microscopy is not always the most reliable method. Therefore, the assessment between conventional PCR and LAMP was also assessed. There was an 80% congruence between the two tests (8/10) with a single sample positive by PCR but negative by LAMP (C3), a single sample negative by PCR but positive by LAMP (A1). Only one sample (A5) did not have sufficient volume to complete a DNA extraction and subsequent conventional PCR.

6.5 Discussion

The accurate and rapid detection of *S. scabiei* in clinical specimens is critical to the appropriate and timely treatment of affected individuals and the implementation of control strategies to reduce the transmission of this parasitic mite. Microscopic examination of skin scrapings continues to be the gold standard for the detection of *S. scabiei* in combination with a detailed clinical assessment. However, comparisons with molecular methods have revealed

Table 6.7: LAMP, microscopy and PCR results of the rapidly processed 11 wombat skin scrapings.

Sample	Microscopy	LAMP	Time to amplify (minutes)	Melt (ºC)
A1	Positive	Positive	17.15	84.96
A3	Positive	Positive	22.15	84.91
A5	Positive	Positive	16.00	84.65
A7	Positive	Positive	13.15	85.20
B1	Negative	Negative		
B3	Positive	Positive	18.15	84.61
B5	Positive	Positive	26.00	84.28
B7	Negative	Negative		
C1	Positive	Positive	22.30	85.07
C3	Negative	Negative		
C5	Negative	Negative		

shortcomings in the sensitivity in the detection of this ectoparasite, particularly in individuals with only low *S. scabiei* mite counts [170, 171]. Building on a growing evidence for the diagnostic utility of NAATs, this study successfully developed a rapid and specific LAMP assay as an ancillary method for detection of *S. scabiei* at the POC.

We demonstrated that the newly designed *S. scabiei* LAMP assay is not just specific and robust, but also capable of providing a rapid diagnostic result (within 30 min). However, much like microscopy and PCR, the result of LAMP is only a reflection within the sample itself rather than the overall health of the individual, the amount of DNA containing a mite in a typical skin scraping does lie to chance. Beyond the increase in speed of this new *S. scabiei* assay compared to a conventional PCR assay, we show that DNA from a single mite can be easily detected in a clinical sample. This level of sensitivity is particularly important given that (a) the mite load is highly variable in individuals compared to their disease presentation, particularly in individuals with early signs of scabies/mange [14]; and (b) the quality and size of the skin scraping is likely to significantly affect the number of mites that will be sampled from the dermis at the affected anatomical site [171]. It has been previously described that even multiple skin scrapings from the same region will harbour varying results for sarcoptic mange. This is not limited to scabies alone, as low parasitic burdens complicate sampling and diagnosis of other parasitic infestations, such as cutaneous leishmaniasis (CL), particularly in post-treatment [184]. LAMP assays on skin biopsies of CL patients were found to be successful prior to treatment, but were unsuccessful in follow-up collections as a result of a low parasite burden [177]. Hence, the sensitivity of detection reported in this newly designed *S. scabiei* LAMP assay is promising. Indeed, the detection of *S. scabiei* DNA in several samples that were negative by our comparative *S. scabiei* conventional PCR assay would suggest that LAMP could be even more sensitive than conventional PCR for *S. scabiei* detection. Potential explanations for this enhanced sensitivity include that (i) the quantity of

amplicons generated by LAMP assays are considerably higher in comparison to those produced by PCR, (ii) due to the use of six specific primers rather than two used in PCR the amplification itself is more targeted, and (iii) the detection of amplicons is by fluorescence rather than visualisation on an agarose gel [185].

A critical aspect limiting the application of this NAAT assay at the POC is the general requirement for a DNA extraction prior to PCR detection. Although our results are preliminary, our data showed that rapid commercial DNA processing kits such as those used in this study appear to be effective in lysing mites embedded in tissues to release DNA for detection. When this method was combined with the LAMP assay itself, it meant that the complete reaction time from sampling to result would be generally around 40 min. Such innovation, alongside alternative sample processing and amplicon detection methods, has the potential to make this *S. scabiei* LAMP assay a POC reality with further development. We and others have also investigated the use of swab sampling of the affected dermis for *S. scabiei* detection [151, 171]. If further validation would reveal that this non-invasive sampling is suitable for *S. scabiei* detection, this approach combined with the LAMP assay would fulfil the requirements for an assay that can be deployed in a range of clinical settings.

In this study we focussed on the ITS-2 gene as a LAMP target, however three *S. scabiei* mitochondrial genes cox1, 12S rRNA and 16S rRNA could be potentially viable targets for LAMP assays based on the availability of sequence data. While not investigated here, we considered but did not pursue these less viable options, as (i) the sequence variation is higher at these mitochondrial loci across the different *S. scabiei* evolutionary lineages and (ii) low GC% content of mitochondrial genes could be problematic for the LAMP assay primer design [128, 146]. Unfortunately, in this study we did not have human *S. scabiei* samples to evaluate with our new assay. Nevertheless, our research [146] and that of others [101-103, 114, 115] shows ITS-2 to be highly conserved across host species and, thus, there is no

evidence to suggest the LAMP assay developed here would not work equally well on *S. scabiei* infesting humans.

Besides detection efficiency and species-specific target, other factors (including cost, time and technical skill) should be also considered during the development of a new diagnostic assay. Regarding scabies, microscopy, although cost efficient, requires technical skill to distinguish mites and eggs within a skin scraping, with a single scraping analysed at a time. Conversely, PCR assays can analyse multiple samples at a time and are highly sensitive at detecting mite DNA, eliminating the requirement for specialised technical skills. However, the time to obtain a result can take up to 24 h and the additional equipment and reagents (i.e., accessible laboratory, PCR and electrophoresis equipment and DNA extraction kits) are essential for this diagnostic method. The LAMP assay, in comparison, can be both cost efficient and rapid, requiring general technical expertise. The fluorometer (such as Genie III used in this study), as one-off cost all-inclusive instrument is portable and can be run off batteries. As the master mix can be pre-aliquoted and the template rapidly processed, the requirement for additional kits and laboratory space is limited. However, further development for a microfluidic device or colorimetry and the efficiency of the rapid DNA extraction is required for this LAMP assay to be classified as a POC test.

6.6 Conclusion

In conclusion, this study describes a development of a new assay for the animal and human *S. scabiei* detection at the POC as well as laboratory. With further development, this assay has the potential to complement existing diagnostic methods in the clinical setting and may offer a low cost, portable option for *S. scabiei* DNA detection in remote or resource-deficient regions.

CHAPTER 7: THESIS SYNTHESIS AND FUTURE DIRECTIONS

7.1 Summary of thesis

The work presented in this thesis has contributed to our knowledge of the genetic composition of *S. scabiei* circulating within Australian wildlife, the likely spill-over events that may have contributed to its origin and, the techniques and methods used for diagnosis. This thesis comprises two major objectives. The first focused on a genetic component of *S. scabiei* which gave emphasis to the molecular typing and epidemiology of this mite infesting key marsupial hosts in Australia, highlighted by significant knowledge gaps and limitations of past phylogenetic research. Chapter 2, Chapter 3 and Chapter 4 progressively determined Australian and global *S. scabiei* mite host specificity and spill-over. The second major objective of this thesis encompasses sarcoptic mange and scabies diagnosis, discussed in Chapter 5 and Chapter 6. These two chapters identified the limitations of an array of commonly used diagnostic methods (Chapter 5) and utilised the outcomes to develop a novel rapid diagnostic assay in Chapter 6.

The major findings reported by this thesis include;

1) evidence to support multiple European and non-European canid and livestock were the source of mange into Australian wildlife. This was associated with an unexpected level of genetic diversity of mites infesting a variety of Australian marsupials.
2) the assessment of various diagnostic methods showing that nucleic acid amplification assays are the most sensitive diagnostic tool for detecting *S. scabiei*.

The endemic disease caused by the *S. scabiei* mite has a significant impact on public and animal health. The availability of epidemiological information and genetic material for *S. scabiei* in Australia has been low, in contrast to the rest of the globe. Prior to this thesis, many of the Australian derived *S. scabiei* studies focused on infestations within remote aboriginal

communities or used pigs as a model for human infestations [60, 127, 186-188]. Microsatellite analysis and targeted single gene phylogenetic analysis of *S. scabiei* collected from humans and dogs from Australia have been investigated, with limited numbers of native animals included [72, 73]. More recently, the effects of sarcoptic mange on Australian wombats and koalas has been a topic for investigation. Sarcoptic mange infestation in these hosts has been associated with behavioural changes, localised population collapse and cellular and pathogenetic responses [38, 40, 74, 81, 168, 189]. Research from this thesis has provided insight into the epidemiological importance of *S. scabiei* within Australia and how these infestations are situated globally. By highlighting the lack of Australian marsupial derived studies and the inconsistencies of phylogenetic gene targets identified, Chapter 2 spurred the focus and direction of my thesis. My research has not only expanded the current genetic repertoire of *S. scabiei* circulating throughout Australia but has also provided the first complete mitochondrial genomes of mites collected from marsupials and emphasised the genetic variation within a given population and between hosts across five states of Australia.

This research has revealed that high genetic variation of *S. scabiei* within Australian marsupials is not a reflection of host species or location, as some haplotypes were identical to other infested animal populations across the globe. This high level of variation is not uncommon and novel to Australia, as the suggestion of multiple strains circulating among a given population has been previously documented in North American black bears [43] and Iberian wolves [190]. This thesis was also able to investigate within-host genetic variation of *S. scabiei* mites. Multiple skin scrapings obtained from different body segments from the same individual generally harboured the same *S. scabiei* haplotype. There were only two wombats what were exceptions, with the differences being due to only a single point mutation between different skin scrapings sites. Past studies using microsatellites, 16S rRNA and *cox1* genes were also unable to differentiate subpopulations by skin site preferences, but was instead indicative of

individual hosts [113, 191]. Overall, this thesis supports the notion that a dominant mite population infests an individual.

Transmission between individuals and spill-over between different host species has been a topic of discussion for two decades [14]. While direct contact with an infested individual is deemed the most plausible mode of mite transmission, indirect transmission is also a possibility, as we now know that the mite can survive off its host in ideal conditions for up to 19 days [14]. In the case for *S. scabiei* infesting Australian wildlife, canid mites are either genetically identical or share a close ancestral lineage with Australian wildlife. This is not surprising as wild dogs and foxes are known to occasionally hunt wombats and koalas, and have been observed to develop mange after hunting or visiting a mangy wombats burrow [58, 59]. However, prior to this thesis, the suggestion that transmission of *S. scabiei* between livestock and Australian marsupials had not been adequately addressed. Whether this spill-over is caused by direct contact between the animals, environmental transmission by "host-jumping" [192] or if there is a third unknown source of transmission (e.g. an unknown reservoir species) requires further research. Furthermore, the similarity of *S. scabiei* infesting Australian wombats and Asian/Egyptian sheep, buffalo and canids cannot completely rule out the possibility that some global infestations originated from Australia also. As many Australian livestock exports are established to Asian and Middle Eastern regions, it is not yet possible to identify directional spill-over, as there is limited knowledge of evolutionary rates for *S. scabiei* mites, and insufficient mitochondrial genetic divergence.

Identical *S. scabiei* haplotypes between hosts leads to limitations in determining the origins of mange in Australia. This thesis largely focused on mitochondrial sequencing of the *S. scabiei* mite. The *cox1* gene used in this thesis, and its corresponding 386bp length, was also selected because of the magnitude of available *S. scabiei* sequences for global assessment. Although the length of gene target was only 24.9% of the full length *cox1* gene, the outcomes from this

thesis did contribute to novel and important observations regarding secondary introduction into Australia and canid and livestock sources. Of course, sequencing the nuclear genome is an obvious future direction to increase our understanding of the molecular epidemiology of *S. scabiei*.

By sequencing the mitochondrial genomes of *S. scabiei* collected from Australian marsupials, I have contributed to the development of genome resources for this mite. Prior to the outcomes obtained in Chapter 3, only a single complete mitochondrial genome was available for a human derived *S. scabiei* mite, and partial draft genomes for pig and dog derived mites. Using these genetic resources, I was nevertheless able to determine that a single gene, *cox1*, could produce a phylogeny that reflected the phylogeny derived from the whole mitochondrial genome analysis. Although this analysis surrounds the mitochondrial genome, future studies surrounding if a nuclear gene, other than the relatively uninformative ITS-2 gene, relates to the mitochondrial phylogeny is be imperative.

The question of whether the genetic predisposition of the host or the mite is a definitive reason for developing crusted or ordinary scabies remains uncertain. The global analysis in Chapter 6 suggests that the genetic differences identified in the mite is unlikely, but rather emphasises the importance of host physiology and immune responses. Although this thesis was investigating the maternally inherited mitochondrial genome, perhaps the argument of disease progression may lie in the relationship between the host immune response and the immune inhibiting factors secreted by the mite [14].

Advanced techniques and correct sampling regimes for *S. scabiei* prove to be critical for future diagnosis and prevention. The global socioeconomic problem and major economic losses associated with the disease resulting from *S. scabiei* infestation requires accurate and timely diagnosis [14, 19]. The difficulty in diagnosing *S. scabiei* does not lie with crusted scabies in

humans and animals with advanced mange, as they present with clear clinical signs and harbour thousands of mites, but rather with early infestation and ordinary scabies [14]. It is well known that the current diagnostic techniques lack sensitivity during early stages of infestation and can result in false negatives, treatment resistance and consequential spread of parasites. The content explored in this thesis surrounding the advantages and disadvantages of microscopy, observational scoring and nucleic acid amplification assays using the bare-nosed wombat has important implications for human and animal *S. scabiei* infestations. Although it would have been ideal to assess antigen-based assays to fully assess all advanced diagnostic techniques, this thesis has rather assessed clinically common techniques and highlighted (i) the necessity for collecting multiple skin scrapings from an individual, (ii) how molecular techniques are more sensitive of detecting mite DNA within a skin scraping, (iii) that microscopy and observational scoring are competent methods for mid to late stage infestations and, (iv) the procedures of which samples are collected are imperative for up-take of mites and their eggs. Accordingly, advancement of diagnoses developed in this thesis has significant implications for surveillance diagnostic tools for both humans and animals and identification of healthy communities for relocation/conservation programs in native Australian wildlife.

Although both Chapter 5 and Chapter 6 identified that NAATS are a more effective method at detecting *S. scabiei* DNA, these methods reflect detection only within the skin scraping collected. As an epidermal parasite known to infest at low numbers and cause hypersensitivity, collection of a skin scraping containing mites, mite eggs or faeces is limited particularly at early stages. This complication has been documented in other parasites, as detection by skin smear microscopy, culture and serological methods are considered problematic due to low parasite burden and antibodies not present in early infestation [176, 177]. In the hopes to detect *S. scabiei* infestations earlier and advance the techniques discussed in this thesis, the delay in symptoms and the mite's ability to produce hypersensitivity needs to be investigated further.

7.2 Future Directions

The research in this thesis embeds well within contemporary One Health principles and illustrates human-wildlife-domestic animal-environmental linkages. Accordingly, *S. scabiei* is an excellent example of a parasite that should not be evaluated in some host taxa, to the exclusion of others, or the environment. Research into the complex interplay of multiple spill-over events, and contemporary assessment of interactions between Australian wildlife, canids and livestock are necessary to establish spill-over and global ancestral lineages of *S. scabiei*. To establish detection of direction of both local and global spill-over and transmission, whole *S. scabiei* genome sequencing, coupled with advanced phylogenetic modelling, is essential across a large variety of hosts. To determine environmental transmission, i.e. within wombat burrows, DNA techniques are necessary for targeted testing. This thesis has shown that isothermal amplification assays are an effective method for a point-of-care test, but to advance it to the point that it can be used in the clinical setting the flowing still needs to be conducted: (i) expand the test to human patients, (ii) further develop the rapid DNA extraction method, (iii) develop a turbidity test which would eliminate the requirement for expensive equipment and, (iv) assess the capabilities of isothermal amplification assays in field and remote settings.

REFERENCES

1. Conti LA, Rabinowitz PM: **One health initiative**. *Infektološki glasnik* 2011, **31**(4):176-178.
2. Zinsstag J, Schelling E, Waltner-Toews D, Tanner M: **From "one medicine" to "one health" and systemic approaches to health and well-being**. *Preventive Veterinary Medicine* 2011, **101**(3):148-156.
3. Woolhouse ME, Gowtage-Sequeria S: **Host range and emerging and reemerging pathogens**. *Emerging Infectious Diseases* 2005, **11**(12):1842.
4. Thompson RA: **Parasite zoonoses and wildlife: one health, spillover and human activity**. *International Journal for Parasitology* 2013, **43**(12):1079-1088.
5. Tompkins DM, Carver S, Jones ME, Krkošek M, Skerratt LF: **Emerging infectious diseases of wildlife: a critical perspective**. *Trends in Parasitology* 2015, **31**(4):149-159.
6. Power AG, Mitchell CE: **Pathogen spillover in disease epidemics**. *The American Naturalist* 2004, **164**(S5):S79-S89.
7. Kennedy S, Kuiken T, Jepson PD, Deaville R, Forsyth M, Barrett T, Van de Bildt M, Osterhaus A, Eybatov T, Duck C: **Mass die-off of Caspian seals caused by canine distemper virus**. *Emerging Infectious Diseases* 2000, **6**(6):637.
8. Alexander KA, Appel MJ: **African wild dogs (*Lycaon pictus*) endangered by a canine distemper epizootic among domestic dogs near the Masai Mara National Reserve, Kenya**. *Journal of Wildlife Diseases* 1994, **30**(4):481-485.
9. Qiu W, Zheng Y, Zhang S, Fan Q, Liu H, Zhang F, Wang W, Liao G, Hu R: **Canine distemper outbreak in rhesus monkeys, China**. *Emerging Infectious Diseases* 2011, **17**(8):1541.

10. Viana M, Cleaveland S, Matthiopoulos J, Halliday J, Packer C, Craft ME, Hampson K, Czupryna A, Dobson AP, Dubovi EJ: **Dynamics of a morbillivirus at the domestic–wildlife interface: Canine distemper virus in domestic dogs and lions**. *Proceedings of the National Academy of Sciences* 2015, **112**(5):1464-1469.

11. Jelocnik M, Frentiu FD, Timms P, Polkinghorne A: **Multilocus sequence analysis provides insights into molecular epidemiology of *Chlamydia pecorum* infections in Australian sheep, cattle, and koalas**. *Journal of Clinical Microbiology* 2013, **51**(8):2625-2632.

12. Halliday R, O'Connor B, Baker A: **Global diversity of mites**. Washington DC: National Academy Press; 2000.

13. Fischer K, Walton S: **Parasitic mites of medical and veterinary importance–is there a common research agenda?** *International Journal for Parasitology* 2014, **44**(12):955-967.

14. Arlian LG, Morgan MS: **A review of *Sarcoptes scabiei*: past, present and future**. *Parasites & Vectors* 2017, **10**(1):297.

15. Klompen J: **Phylogenetic relationships in the mite family *Sarcoptidae* (Acari: Astigmata)**. 1992.

16. Mellanby K: **The development of symptoms, parasitic infection and immunity in human scabies**. *Parasitology* 1944, **35**(4).

17. Munro J: **Report of Scabies Investigation**. *Journal of the Royal Army Medical Corps* 1919, **33**(1).

18. Skerratt LF, Middleton D, Beveridge I: **Distribution of life cycle stages of *Sarcoptes scabiei* var *wombati* and effects of severe mange on common wombats in Victoria**. *Journal of Wildlife Diseases* 1999, **35**(4):633-646.

19. Walton SF, Currie BJ: **Problems in diagnosing scabies, a global disease in human and animal populations**. *Clinical Microbiology Reviews* 2007, **20**(2):268-279.

20. Pence D, Ueckermann E: **Sarcoptic manage in wildlife**. *Revue scientifique et technique (International Office of Epizootics)* 2002, **21**(2):385-398.

21. Wendel K, Rompalo A: **Scabies and pediculosis pubis: an update of treatment regimens and general review**. *Clinical Infectious Diseases* 2002, **35**(Supplement_2):S146-S151.

22. Bhat SA, Mounsey KE, Liu X, Walton SF: **Host immune responses to the itch mite, *Sarcoptes scabiei*, in humans**. *Parasites & Vectors* 2017, **10**(1):385.

23. McCarthy J, Kemp DJ, Walton SF, Currie BJ: **Scabies: more than just an irritation**. *Postgraduate Medical Journal* 2004, **80**(945):382-387.

24. Walton S: **The immunology of susceptibility and resistance to scabies**. *Parasite Immunology* 2010, **32**(8):532-540.

25. Olivier M, Gregory DJ, Forget G: **Subversion mechanisms by which *Leishmania* parasites can escape the host immune response: a signaling point of view**. *Clinical Microbiology Reviews* 2005, **18**(2):293-305.

26. Denkers EY: **From cells to signaling cascades: manipulation of innate immunity by *Toxoplasma gondii***. *FEMS Immunology & Medical Microbiology* 2003, **39**(3):193-203.

27. Morgan MS, Arlian LG, Markey MP: ***Sarcoptes scabiei* mites modulate gene expression in human skin equivalents**. *PLoS One* 2013, **8**(8):e71143.

28. Wilson A: **Immune responses to ectoparasites of horses, with a focus on insect bite hypersensitivity**. *Parasite Immunology* 2014, **36**(11):560-572.

29. Sarre C, González-Hernández A, Coppernolle S, Grit R, Grauwet K, Meulder F, Chiers K, den Broeck W, Geldhof P, Claerebout E: **Comparative immune responses**

against Psoroptes ovis in two cattle breeds with different susceptibility to mange. *Veterinary Research* 2015, **46**(1):131.

30. Nicholls SG, Oakley CL, Tan A, Vote BJ: **Demodex species in human ocular disease: new clinicopathological aspects**. *International Ophthalmology* 2017, **37**(1):303-312.

31. Mellanby K: **Scabies in 1976**. *Royal Society of Health Journal* 1977, **97**(1):32.

32. Arlian LG: **Biology, host relations, and epidemiology of *Sarcoptes scabiei***. *Annual Review of Entomology* 1989, **34**(1):139-159.

33. Davis DP, Moon RD: **Density of itch mite, *Sarcoptes scabiei* (Acari: Sarcoptidae) and temporal development of cutaneous hypersensitivity in swine mange**. *Veterinary parasitology* 1990, **36**(3):285-293.

34. Skerratt LF: ***Sarcoptes scabiei*: an important exotic pathogen of wombats**. *Microbiolgy Australia* 2005, **26**(2):79-81.

35. Old J, Sengupta C, Narayan E, Wolfenden J: **Sarcoptic mange in wombats—A review and future research directions**. *Transboundary and Emerging Diseases* 2018, **65**(2):399-407.

36. Leung V, Miller M: **Detection of scabies: A systematic review of diagnostic methods**. *Canadian Journal of Infectious Diseases and Medical Microbiology* 2011, **22**(4):143-146.

37. Almberg ES, Cross PC, Dobson AP, Smith DW, Hudson PJ: **Parasite invasion following host reintroduction: a case study of Yellowstone's wolves**. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* 2012, **367**(1604):2840-2851.

38. Martin AM, Burridge CP, Ingram J, Fraser TA, Carver S: **Invasive pathogen drives host population collapse: effects of a travelling wave of sarcoptic mange on bare-nosed wombats**. *Journal of Applied Ecology* 2018, **55**:331–341.

39. Samuel WMP, M.J. and Kocan, A.A: **Parasitic Diseases of Wild Mammals**, 2nd edn. Iowa State University Press2001.

40. Simpson K, Johnson CN, Carver S: ***Sarcoptes scabiei*: The Mange Mite with Mighty Effects on the Common Wombat (*Vombatus ursinus*)**. *PLoS One* 2016, **11**(3):e0149749.

41. Turchetto S, Obber F, Permunian R, Vendrami S, Lorenzetto M, Ferré N, Stancampiano L, Rossi L, Citterio CV: **Spatial and temporal explorative analysis of sarcoptic mange in Alpine chamois (*Rupicapra r. rupicapra*)**. *Hystrix, the Italian Journal of Mammalogy* 2014, **25**(1):25-30.

42. Samuel W: **Attempted experimental transfer of sarcoptic mange (*Sarcoptes scabiei*, Acarina: Sarcoptidae) among red fox, coyote, wolf and dog**. *Journal of Wildlife Diseases* 1981, **17**(3):343-347.

43. Peltier SK, Brown JD, Ternent M, Niedringhaus KD, Schuler K, Bunting EM, Kirchgessner M, Yabsley MJ: **Genetic Characterization of *Sarcoptes scabiei* from Black Bears (*Ursus americanus*) and Other Hosts in the Eastern United States**. *Journal of Parasitology* 2017, **103**(5):593-597.

44. Shen N, He R, Liang Y, Xu J, He M, Ren Y, Gu X, Lai W, Xie Y, Peng X: **Expression and characterisation of a *Sarcoptes scabiei* protein tyrosine kinase as a potential antigen for scabies diagnosis**. *Scientific Reports* 2017, **7**(1):9639.

45. Bornstein S, Frössling J, Näslund K, Zakrisson G, Mörner T: **Evaluation of a serological test (indirect ELISA) for the diagnosis of sarcoptic mange in red foxes (*Vulpes vulpes*)**. *Veterinary Dermatology* 2006, **17**(6):411-416.

46. Little SE, Davidson WR, Rakich PM, Nixon TL, Bounous DI, Nettles VF: **Responses of red foxes to first and second infection with Sarcoptes scabiei**. *Journal of wildlife diseases* 1998, **34**(3):600-611.

47. Sarasa M, Rambozzi L, Rossi L, Meneguz PG, Serrano E, Granados J-E, González FJ, Fandos P, Soriguer RC, Gonzalez G: **Sarcoptes scabiei: Specific immune response to sarcoptic mange in the Iberian ibex Capra pyrenaica depends on previous exposure and sex**. *Experimental parasitology* 2010, **124**(3):265-271.

48. Arlian LG, Feldmeier H, Morgan MS: **The potential for a blood test for scabies**. *PLoS Neglected Tropical Diseases* 2015, **9**(10):e0004188.

49. Curtis CF: **Current trends in the treatment of Sarcoptes, Cheyletiella and Otodectes mite infestations in dogs and cats**. *Veterinary Dermatology* 2004, **15**(2):108-114.

50. Haas N, Wagemann B, Hermes B, Henz BM, Heile C, Schein E: **Crossreacting IgG antibodies against fox mite antigens in human scabies**. *Archives for Dermatological Research* 2005, **296**(7):327-331.

51. Rodríguez-Cadenas F, Carbajal-González M, Fregeneda-Grandes J, Aller-Gancedo J, Huntley J, Rojo-Vázquez F: **Development and evaluation of an antibody ELISA for sarcoptic mange in sheep and a comparison with the skin-scraping method**. *Preventive Veterinary Medicine* 2010, **96**(1-2):82-92.

52. Rambozzi L, Menzano A, Lavin S, Rossi L: **Biotin-avidin amplified ELISA for detection of antibodies to *Sarcoptes scabiei* in chamois (*Rupicapra* spp.)**. *Veterinary Research* 2004, **35**(6):701-708.

53. Casais R, Prieto M, Balseiro A, Solano P, Parra F, Alonso JMM: **Identification and heterologous expression of a *Sarcoptes scabiei* cDNA encoding a structural**

antigen with immunodiagnostic potential. *Veterinary Research* 2007, **38**(3):435-450.

54. Millán J, Casáis R, Delibes-Mateos M, Calvete C, Rouco C, Castro F, Colomar V, Casas-Díaz E, Ramírez E, Moreno S: **Widespread exposure to *Sarcoptes scabiei* in wild European rabbits (*Oryctolagus cuniculus*) in Spain**. *Veterinary parasitology* 2012, **183**(3-4):323-329.

55. He R, Shen N, Lin H, Gu X, Lai W, Peng X, Yang G: **Molecular characterization of calmodulin from *Sarcoptes scabiei***. *Parasitology International* 2017, **66**(2):1-6.

56. Mattsson J, Ljunggren E, Bergström K: **Paramyosin from the parasitic mite *Sarcoptes scabiei*: cDNA cloning and heterologous expression**. *Parasitology* 2001, **122**(5):555-562.

57. Arlian L, Runyan R, Achar S, Estes S: **Survival and infestivity of *Sarcoptes scabiei* var. *canis* and var. *hominis***. *Journal of the American Academy of Dermatology* 1984, **11**(2):210-215.

58. Gray D: **Sarcoptic mange affecting wild fauna in New South Wales**. *Australian Veterinary Journal* 1937, **13**(4):154-155.

59. Skerratt L, Martin R, Handasyde K: **Sarcoptic mange in wombats**. *Australian Veterinary Journal* 1998, **76**(6):408-410.

60. Mounsey KE, Murray HC, Bielefeldt-Ohmann H, Pasay C, Holt DC, Currie BJ, Walton SF, McCarthy JS: **Prospective Study in a Porcine Model of *Sarcoptes scabiei* Indicates the Association of Th2 and Th17 Pathways with the Clinical Severity of Scabies**. *PLoS Neglected Tropical Diseases* 2015, **9**(3):e0003498.

61. Alasaad S, Walton S, Rossi L, Bornstein S, Abu-Madi M, Soriguer RC, Fitzgerald S, Zhu X-Q, Zimmermann W, Ugbomoiko US: ***Sarcoptes*-World molecular network**

(*Sarcoptes*-WMN): Integrating research on scabies. *International Journal of Infectious Diseases* 2011, **15**(5):e294-e297.

62. Skerratt LF, Beveridge I: **Human scabies of wombat origin**. *Australian Veterinary Journal* 1999, **77**(9):607-607.

63. Alasaad S, Rossi L, Heukelbach J, Pérez JM, Hamarsheh O, Otiende M, Zhu X-Q: **The neglected navigating web of the incomprehensibly emerging and re-emerging *Sarcoptes* mite**. *Infection, Genetics and Evolution* 2013, **17**:253-259.

64. Estes SA, Kummel B, Arlian L: **Experimental canine scabies in humans**. *Journal of the American Academy of Dermatology* 1983, **9**(3):397-401.

65. Chitty J, Hendricks A: **Zoonotic skin disease in small animals**. *IN PRACTICE-LONDON-BRITISH VETERINARY ASSOCIATION-* 2007, **29**(2):92.

66. Diwakar R, Diwakar R: **Canine scabies: a zoonotic ectoparasitic skin disease**. *Int J Curr Microbiol Appl Sci* 2017, **6**:1361-1365.

67. Andriantsoanirina V, Ariey F, Izri A, Bernigaud C, Fang F, Charrel R, Foulet F, Botterel F, Guillot J, Chosidow O: ***Sarcoptes scabiei* mites in humans are distributed into three genetically distinct clades**. *Clinical Microbiology and Infection* 2015, **21**(12):1107-1114.

68. Rider SD, Morgan MS, Arlian LG: **Draft genome of the scabies mite**. *Parasites & Vectors* 2015, **8**(1):585.

69. Mofiz E, Holt DC, Seemann T, Currie BJ, Fischer K, Papenfuss AT: **Genomic resources and draft assemblies of the human and porcine varieties of scabies mites, *Sarcoptes scabiei* var. *hominis* and var. *suis***. *GigaScience* 2016, **5**(1):1.

70. Berrilli F, D'Amelio S, Rossi L: **Ribosomal and mitochondrial DNA sequence variation in *Sarcoptes* mites from different hosts and geographical regions**. *Parasitology Research* 2002, **88**(8):772-777.

71. Soglia D, Rasero R, Rossi L, Sartore S, Sacchi P, Maione S: **Microsatellites as markers for comparison among different populations of *Sarcoptes scabiei***. *Italian Journal of Animal Science* 2007, **6**(1s):214-216.

72. Walton S, Dougall A, Pizzutto S, Holt D, Taplin D, Arlian L, Morgan M, Currie B, Kemp D: **Genetic epidemiology of *Sarcoptes scabiei* (Acari: *Sarcoptidae*) in northern Australia**. *International Journal for Parasitology* 2004, **34**(7):839-849.

73. Walton SF, Choy JL, Bonson A, Valle A, McBroom J, Taplin D, Arlian L, Mathews JD, Currie B, Kemp DJ: **Genetically distinct dog-derived and human-derived *Sarcoptes scabiei* in scabies-endemic communities in northern Australia**. *The American Journal of Tropical Medicine and Hygiene* 1999, **61**(4):542-547.

74. Speight K, Whiteley P, Woolford L, Duignan P, Bacci B, Lathe S, Boardman W, Scheelings T, Funnell O, Underwood G: **Outbreaks of sarcoptic mange in free-ranging koala populations in Victoria and South Australia: a case series**. *Australian Veterinary Journal* 2017, **95**(7):244-249.

75. McLelland D, Youl J: **Sarcoptic mange in agile wallabies (*Macropus agilis*) in the Northern Territory**. *Australian Veterinary Journal* 2005, **83**(12):744-745.

76. Holz PH, Orbell GMB, Beveridge I: **Sarcoptic mange in a wild swamp wallaby (*Wallabia bicolor*)**. *Australian Veterinary Journal* 2011, **89**(11):458-459.

77. Wicks R, Clark P, Hobbs R: **Clinical dermatitis in a southern brown bandicoot (*Isoodon obesulus*) associated with the mite *Sarcoptes scabiei***. *Comparative Clinical Pathology* 2007, **16**(4):271-274.

78. McCarthy P: **The presence of sarcoptic mange in the wild fox (*Vulpes vulpes*) in Central Queensland**. *Australian Veterinary Journal* 1960, **36**(8):359-360.

79. McCarthy P: **The transmission of sarcoptic mange from the wild fox (*Vulpes vulpes*) to man and other species in central Queensland**. *Australian Veterinary Journal* 1960, **36**(12):479-480.

80. Ruykys L, Taggart DA, Breed WG, Schultz D: **Sarcoptic mange in southern hairy-nosed wombats (*Lasiorhinus latifrons*): distribution and prevalence in the Murraylands of South Australia**. *Australian Journal of Zoology* 2009, **57**(2):129-138.

81. Skerratt LF: **Cellular response in the dermis of common wombats (*Vombatus ursinus*) infected with *Sarcoptes scabiei* var. *wombati***. *Journal of Wildlife Diseases* 2003, **39**(1):193-202.

82. Skerratt L, Campbell N, Murrell A, Walton S, Kemp D, Barker S: **The mitochondrial 12S gene is a suitable marker of populations of *Sarcoptes scabiei* from wombats, dogs and humans in Australia**. *Parasitology Research* 2002, **88**(4):376-379.

83. Johnson PT, De Roode JC, Fenton A: **Why infectious disease research needs community ecology**. *Science* 2015, **349**(6252):1259504.

84. Cleaveland S, Laurenson M, Taylor L: **Diseases of humans and their domestic mammals: pathogen characteristics, host range and the risk of emergence**. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* 2001, **356**(1411):991-999.

85. Firth C, Lipkin WI: **The genomics of emerging pathogens**. *Annual Review of Genomics and Human Genetics* 2013, **14**:281-300.

86. Morse SS, Mazet JA, Woolhouse M, Parrish CR, Carroll D, Karesh WB, Zambrana-Torrelio C, Lipkin WI, Daszak P: **Prediction and prevention of the next pandemic zoonosis**. *The Lancet* 2012, **380**(9857):1956-1965.

87. Mounsey KE, McCarthy JS, Walton SF: **Scratching the itch: new tools to advance understanding of scabies**. *Trends in Parasitology* 2012, **29**(1):35-42.

88. Serrano E, Colom-Cadena A, Gilot-Fromont E, Garel M, Cabezón O, Velarde R, Fernández-Sirera L, Fernández-Aguilar X, Rosell R, Lavín S: **Border Disease Virus: an exceptional driver of chamois populations among other threats**. *Frontiers in Microbiology* 2015, **6**.

89. Daszak P, Cunningham AA, Hyatt AD: **Emerging infectious diseases of wildlife--threats to biodiversity and human health**. *Science* 2000, **287**(5452):443-449.

90. Alasaad S, Ndeereh D, Rossi L, Bornstein S, Permunian R, Soriguer RC, Gakuya F: **The opportunistic *Sarcoptes scabiei*: A new episode from giraffe in the drought-suffering Kenya**. *Veterinary parasitology* 2012, **185**(2):359-363.

91. Alasaad S, Permunian R, Gakuya F, Mutinda M, Soriguer RC, Rossi L: **Sarcoptic-mange detector dogs used to identify infected animals during outbreaks in wildlife**. *BMC Veterinary Research* 2012, **8**(1):110.

92. Obendorf DL: **Causes of mortality and morbidity of wild koalas, *Phascolarctos cinereus* (Goldfuss), in Victoria, Australia**. *Journal of Wildlife Diseases* 1983, **19**(2):123-131.

93. Saunders GR, Gentle MN, Dickman CR: **The impacts and management of foxes *Vulpes vulpes* in Australia**. *Mammal Review* 2010, **40**(3):181-211.

94. Arlian L, Vyszenski-Moher D, Pole M: **Survival of adults and developmental stages of *Sarcoptes scabiei* var. *canis* when off the host**. *Experimental and Applied Acarology* 1989, **6**(3):181-187.

95. Roger E, Laffan SW, Ramp D: **Road impacts a tipping point for wildlife populations in threatened landscapes**. *Popul Ecol* 2011, **53**(1):215-227.

96. Martin R, Handasyde K, Skerratt L: **Current distribution of sarcoptic mange in wombats**. *Australian Veterinary Journal* 1998, **76**(6):411-414.

97. Andrews RM, Kearns T, Connors C, Parker C, Carville K, Currie BJ, Carapetis JR: **A regional initiative to reduce skin infections amongst aboriginal children living in remote communities of the Northern Territory, Australia**. *PLoS Neglected Tropical Diseases* 2009, **3**(11):e554.

98. Al-Musawi MM, Hasan HR, Maluki AH: **Relationship between TH1, TH2 Immune Responses and Serum SOD Activity In Scabies**. *Journal of Advanced Biomedical & Pathobiology Research* 2014, **4**(1):1-15.

99. Walton SF, Pizzutto S, Slender A, Viberg L, Holt D, Hales BJ, Kemp DJ, Currie BJ, Rolland JM, O'Hehir R: **Increased Allergic Immune Response to *Sarcoptes scabiei* Antigens in Crusted versus Ordinary Scabies**. *Clinical and Vaccine Immunology* 2010, **17**(9):1428-1438.

100. Zhang R, Jise Q, Zheng W, Ren Y, Nong X, Wu X, Gu X, Wang S, Peng X, Lai S: **Characterization and evaluation of a *Sarcoptes scabiei* allergen as a candidate vaccine**. *Parasites & Vectors* 2012, **5**(1):1-9.

101. Amer S, El Wahab TA, Metwaly AEN, Ye J, Roellig D, Feng Y, Xiao L: **Preliminary molecular characterizations of *Sarcoptes scaibiei* (Acari: *Sarcoptidae*) from farm animals in Egypt**. *PLoS One* 2014, **9**(4):e94705.

102. Andriantsoanirina V, Ariey F, Izri A, Bernigaud C, Fang F, Guillot J, Chosidow O, Durand R: **Wombats acquired scabies from humans and/or dogs from outside Australia**. *Parasitology Research* 2015.

103. Alasaad S, Soglia D, Spalenza V, Maione S, Soriguer RC, Perez JM, Rasero R, Degiorgis MP, Nimmervoll H, Zhu XQ *et al*: **Is ITS-2 rDNA suitable marker for**

genetic characterization of *Sarcoptes* mites from different wild animals in different geographic areas? *Veterinary parasitology* 2009, **159**(2):181-185.

104. Simon C: **Molecular systematics at the species boundary: exploiting conserved and variable regions of the mitochondrial genome of animals via direct sequencing from amplified DNA**. In: *Molecular Techniques in Taxonomy*. Heidelberg: Springer; 1991: 33-71.

105. Hebert PD, Ratnasingham S, de Waard JR: **Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species**. *Proceedings of the Royal Society of London B: Biological Sciences* 2003, **270**(Suppl 1):S96-S99.

106. Balloux F, Lugon-Moulin N: **The estimation of population differentiation with microsatellite markers**. *Molecular Ecology* 2002, **11**(2):155-165.

107. Zhao Y, Cao Z, Cheng J, Hu L, Ma J, Yang Y, Wang X, Zeng J, Wang T: **Population identification of *Sarcoptes hominis* and *Sarcoptes canis* in China using DNA sequences**. *Parasitology Research* 2014:1-10.

108. Currier RW, Walton SF, Currie BJ: **Scabies in animals and humans: history, evolutionary perspectives, and modern clinical management**. *Annals of the New York Academy of Sciences* 2011, **1230**(1):E50-E60.

109. Alasaad S, Sarasa M, Heukelbach J, Mijele D, Soriguer RC, Zhu X-Q, Rossi L: **Advances in studies of disease-navigating webs: *Sarcoptes scabiei* as a case study**. *Parasites & Vectors* 2014, **7**(1):16.

110. Rasero R, Rossi L, Soglia D, Maione S, Sacchi P, Rambozzi L, Sartore S, Soriguer RC, Spalenza V, Alasaad S: **Host taxon-derived *Sarcoptes* mite in European wild animals revealed by microsatellite markers**. *Biological Conservation* 2010, **143**(5):1269-1277.

111. Alasaad S, Oleaga Á, Casais R, Rossi L, Molinar-Min A, Soriguer RC, Gortázar C: **Temporal stability in the genetic structure of *Sarcoptes scabiei* under the host-taxon law: empirical evidences from wildlife-derived *Sarcoptes* mite in Asturias, Spain**. *Parasites & Vectors* 2011, **4**:151.

112. Gakuya F, Rossi L, Ombui J, Maingi N, Muchemi G, Ogara W, Soriguer RC, Alasaad S: **The curse of the prey: *Sarcoptes* mite molecular analysis reveals potential prey-to-predator parasitic infestation in wild animals from Masai Mara, Kenya**. *Parasites & Vectors* 2011, **4**:193.

113. Makouloutou P, Suzuki K, Yokoyama M, Takeuchi M, Yanagida T, Sato H: **Involvement of two genetic lineages of *Sarcoptes scabiei* mites in a local mange epizootic of wild mammals in Japan**. *Journal of Wildlife Diseases* 2015, **51**(1):69-78.

114. Zahler M, Essig A, Gothe R, Rinder H: **Molecular analyses suggest monospecificity of the genus *Sarcoptes* (Acari: Sarcoptidae)**. *International Journal for Parasitology* 1999, **29**(5):759-766.

115. Gu X-B, Yang G-Y: **A study on the genetic relationship of mites in the genus *Sarcoptes* (Acari: *Sarcoptidae*) in China**. *International Journal of Acarology* 2008, **34**(2):183-190.

116. Erster O, Roth A, Pozzi PS, Bouznach A, Shkap V: **First detection of *Sarcoptes scabiei* from domesticated pig (*Sus scrofa*) and genetic characterization of *S. scabiei* from pet, farm and wild hosts in Israel**. *Experimental and Applied Acarology* 2015, **66**(4):1-8.

117. Huson DH, Bryant D: **Application of phylogenetic networks in evolutionary studies**. *Molecular Biology and Evolution* 2006, **23**(2):254-267.

118. Cameron SL: **Insect mitochondrial genomics: implications for evolution and phylogeny**. *Annual Review of Entomology* 2014, **59**:95-117.

119. Mofiz E, Seemann T, Bahlo M, Holt D, Currie BJ, Fischer K, Papenfuss AT: **Mitochondrial Genome Sequence of the Scabies Mite Provides Insight into the Genetic Diversity of Individual Scabies Infections**. *PLoS Neglected Tropical Diseases* 2016, **10**(2):e0004384.

120. Hayman DT, McDonald KD, Kosoy MY: **Evolutionary history of rat-borne *Bartonella*: the importance of commensal rats in the dissemination of bacterial infections globally**. *Ecology and Evolution* 2013, **3**(10):3195-3203.

121. Morens DM, Folkers GK, Fauci AS: **The challenge of emerging and re-emerging infectious diseases**. *Nature* 2004, **430**(6996):242-249.

122. Bachmann NL, Fraser TA, Bertelli C, Jelocnik M, Gillett A, Funnell O, Flanagan C, Myers GS, Timms P, Polkinghorne A: **Comparative genomics of koala, cattle and sheep strains of *Chlamydia pecorum***. *BMC Genomics* 2014, **15**(1):667.

123. Polkinghorne A, Hanger J, Timms P: **Recent advances in understanding the biology, epidemiology and control of chlamydial infections in koalas**. *Veterinary Microbiology* 2013, **165**(3):214-223.

124. Vos T, Flaxman AD, Naghavi M, Lozano R, Michaud C, Ezzati M, Shibuya K, Salomon JA, Abdalla S, Aboyans V: **Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010**. *The Lancet* 2013, **380**(9859):2163-2196.

125. Walton SF, Holt DC, Currie BJ, Kemp DJ: **Scabies: new future for a neglected disease**. *Advances in Parasitology* 2004, **57**:309-376.

126. Munoz E, Powers JR, Nienhuys TG, Mathews JD: **Social and environmental factors in 10 aboriginal communities in the Northern Territory: relationship to hospital admissions of children**. *Medical Journal of Australia* 1992, **156**(8):529-533.

127. Currie BJ, Carapetis JR: **Skin infections and infestations in Aboriginal communities in northern Australia**. *Australasian Journal of Dermatology* 2000, **41**(3):139-143.

128. Fraser TA, Charleston M, Martin A, Polkinghorne A, Carver S: **The emergence of sarcoptic mange in Australian wildlife: an unresolved debate**. *Parasites & Vectors* 2016, **9**(1):1-11.

129. Mandal SD, Chhakchhuak L, Gurusubramanian G, Kumar NS: **Mitochondrial markers for identification and phylogenetic studies in insects–A Review**. *DNA Barcodes* 2014, **2**(1).

130. Andriantsoanirina V, Fang F, Ariey F, Izri A, Foulet F, Botterel F, Bernigaud C, Chosidow O, Huang W, Guillot J: **Are humans the initial source of canine mange?** *Parasites & Vectors* 2016, **9**(1):177.

131. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C: **Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data**. *Bioinformatics* 2012, **28**(12):1647-1649.

132. Edgar RC: **MUSCLE: multiple sequence alignment with high accuracy and high throughput**. *Nucleic Acids Research* 2004, **32**(5):1792-1797.

133. Laslett D, Canback B: **ARWEN: a program to detect tRNA genes in metazoan mitochondrial nucleotide sequences**. *Bioinformatics* 2008, **24**(2):172-175.

134. Benson DA, Cavanaugh M, Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW: **GenBank**. *Nucleic Acids Research* 2013, **41**(D1):D36-D42.

135. Rozas J: **DnaSP v5: A software for comprehensive analysis of DNA polymorphism data**. *Bioinformatics* 2009, **25**:337-350.

136. Huelsenbeck JP, Ronquist F: **MRBAYES: Bayesian inference of phylogenetic trees**. *Bioinformatics* 2001, **17**(8):754-755.

137. Plomley NJB: **Friendly Mission. The Tasmanian Journals and Papers of George Augustus Robinson 1829-1834**: Queen Victoria Museum and Art Gallery; 2008.

138. Powell M: **Mosquito Brutality and Exile. Aboriginal Resistance in New South Wales and Van Diemen's Land**: Fullers Bookshop Pty Ltd; 2016.

139. Brower A: **Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution**. *Proceedings of the National Academy of Sciences* 1994, **91**(14):6491-6495.

140. Palopoli MF, Fergus DJ, Minot S, Pei DT, Simison WB, Fernandez-Silva I, Thoemmes MS, Dunn RR, Trautwein M: **Global divergence of the human follicle mite *Demodex folliculorum*: Persistent associations between host ancestry and mite lineages**. *Proceedings of the National Academy of Sciences* 2015, **112**(52):15958-15963.

141. Vila M, Björklund M: **The utility of the neglected mitochondrial control region for evolutionary studies in Lepidoptera (Insecta)**. *Journal of Molecular Evolution* 2004, **58**(3):280-290.

142. Arif I, Khan H: **Molecular markers for biodiversity analysis of wildlife animals: a brief review**. *Animal Biodiversity and Conservation* 2009, **32**(1):9-17.

143. Arlian LG, Runyan RA, Estes SA: **Cross infestivity of *Sarcoptes scabiei***. *Journal of the American Academy of Dermatology* 1984, **10**(6):979-986.

144. Oleaga Á, Casais R, Balseiro A, Espí A, Llaneza L, Hartasánchez A, Gortázar C: **New techniques for an old disease: sarcoptic mange in the Iberian wolf**. *Veterinary parasitology* 2011, **181**(2):255-266.

145. Devenish-Nelson ES, Richards SA, Harris S, Soulsbury C, Stephens PA: **Demonstrating frequency-dependent transmission of sarcoptic mange in red foxes**. *Biology letters* 2014, **10**(10):20140524.

146. Fraser TA, Shao R, Fountain-Jones NM, Charleston M, Martin A, Whiteley P, Holme R, Carver S, Polkinghorne A: **Mitochondrial genome sequencing reveals potential origins of the scabies mite *Sarcoptes scabiei* infesting two iconic Australian marsupials**. *BMC Evolutionary Biology* 2017, **17**(1):233.

147. Hadfield J, Croucher NJ, Goater RJ, Abudahab K, Aanensen DM, Harris SR: **Phandango: an interactive viewer for bacterial population genomics**. *Bioinformatics* 2017, **34**(2):292-293.

148. Matsuyama R, Yabusaki T, Kuninaga N, Morimoto T, Okano T, Suzuki M, Asano M: **Coexistence of two different genotypes of *Sarcoptes scabiei* derived from companion dogs and wild raccoon dogs in Gifu, Japan: The genetic evidence for transmission between domestic and wild canids**. *Veterinary parasitology* 2015, **212**(3):356-360.

149. Engelman D, Kiang K, Chosidow O, McCarthy J, Fuller C, Lammie P, Hay R, Steer A, Scabies motIAftCo: **Toward the global control of human scabies: introducing the International Alliance for the Control of Scabies**. *PLoS Neglected Tropical Diseases* 2013, **7**(8):e2167.

150. Hicks MI, Elston DM: **Scabies**. *Dermatology and Therapy* 2009, **22**(4):279-292.

151. Wong SS, Poon RW, Chau S, Wong SC, To KK, Cheng VC, Fung KS, Yuen K: **Development of conventional and realtime quantitative polymerase chain**

reaction assay in the diagnosis and monitoring of scabies. *Journal of Clinical Microbiology* 2015:JCM. 00073-00015.

152. Ladds P: **Pathology of Australian native wildlife**: CSIRO PUBLISHING; 2009.

153. Shojima T, Yoshikawa R, Hoshino S, Shimode S, Nakagawa S, Ohata T, Nakaoka R, Miyazawa T: **Identification of a novel subgroup of koala retrovirus from koalas in Japanese zoos**. *Journal of Virology* 2013, **87**(17):9943-9948.

154. Mackinnon A: **A spreadsheet for the calculation of comprehensive statistics for the assessment of diagnostic tests and inter-rater agreement**. *Computers in Biology and Medicine* 2000, **30**(3):127-134.

155. Team RC: **R foundation for statistical computing**. *Vienna, Austria* 2013, **3**(0).

156. Thompson M, Engelman D, Gholam K, Fuller L, Steer A: **Systematic review of the diagnosis of scabies in therapeutic trials**. *Clinical and Experimental Dermatology* 2017.

157. Cross PC, Almberg ES, Haase CG, Hudson PJ, Maloney SK, Metz MC, Munn AJ, Nugent P, Putzeys O, Stahler DR: **Energetic costs of mange in wolves estimated from infrared thermography**. *Ecology* 2016, **97**(8):1938-1948.

158. Alasaad S, Rossi L, Soriguer RC, Rambozzi L, Soglia D, Pérez JM, Zhu X-Q: **Sarcoptes mite from collection to DNA extraction: the lost realm of the neglected parasite**. *Parasitology Research* 2009, **104**(4):723-732.

159. Bezold G, Lange M, Schiener R, Palmedo G, Sander C, Kerscher M, Peter R: **Hidden scabies: diagnosis by polymerase chain reaction**. *British Journal of Dermatology* 2001, **144**(3):614-618.

160. Fukuyama S, Nishimura T, Yotsumoto H, Gushi A, Tsuji M, Kanekura T, Matsuyama T: **Diagnostic usefulness of a nested polymerase chain reaction assay for**

detecting *Sarcoptes scabiei* DNA in skin scrapings from clinically suspected scabies. *British Journal of Dermatology* 2010, **163**(4):892-894.

161. Maffert P, Reverchon S, Nasser W, Rozand C, Abaibou H: **New nucleic acid testing devices to diagnose infectious diseases in resource-limited settings**. *European Journal of Clinical Microbiology & Infectious Diseases* 2017:1-15.

162. Lower KS, Medleau LM, Hnilica K, Bigler B: **Evaluation of an enzyme-linked immunosorbant assay (ELISA) for the serological diagnosis of sarcoptic mange in dogs**. *Veterinary Dermatology* 2001, **12**(6):315-320.

163. Zheng Y, He R, He M, Gu X, Wang T, Lai W, Peng X, Yang G: **Characterization of *Sarcoptes scabiei* cofilin gene and assessment of recombinant cofilin protein as an antigen in indirect-ELISA for diagnosis**. *BMC Infectious Diseases* 2016, **16**(1):1.

164. Hay RJ, Johns NE, Williams HC, Bolliger IW, Dellavalle RP, Margolis DJ, Marks R, Naldi L, Weinstock MA, Wulf SK: **The global burden of skin disease in 2010: an analysis of the prevalence and impact of skin conditions**. *Journal of Investigative Dermatology* 2014, **134**(6):1527-1534.

165. Romani L, Steer AC, Whitfeld MJ, Kaldor JM: **Prevalence of scabies and impetigo worldwide: a systematic review**. *The Lancet Infectious Diseases* 2015, **15**(8):960-967.

166. Forchhammer MC, Asferg T: **Invading parasites cause a structural shift in red fox dynamics**. *Proceedings of the Royal Society of London B: Biological Sciences* 2000, **267**(1445):779-786.

167. Graczyk TK, Mudakikwa AB, Cranfield MR, Eilenberger U: **Hyperkeratotic mange caused by *Sarcoptes scabiei* (Acariformes: *Sarcoptidae*) in juvenile human-habituated mountain gorillas (*Gorilla gorilla beringei*)**. *Parasitology Research* 2001, **87**(12):1024-1028.

168. Martin AM, Fraser TA, Lesku JA, Simpson K, Roberts GL, Garvey J, Polkinghorne A, Burridge CP, Carver S: **The cascading pathogenic consequences of *Sarcoptes scabiei* infection that manifest in host disease**. *Royal Society Open Science* 2018, **5**(4).

169. Perrucci S, Verin R, Mancianti F, Poli A: **Sarcoptic mange and other ectoparasitic infections in a red fox (*Vulpes vulpes*) population from central Italy**. *Parasite Epidemiology and Control* 2016, **1**(2):66-71.

170. Hardy M, Engelman D, Steer A: **Scabies: A clinical update**. *Australian Family Physician* 2017, **46**(5):264.

171. Fraser TA, Martin A, Polkinghorne A, Carver S: **Comparative diagnostics reveals PCR assays on skin scrapings is the most reliable method to detect *Sarcoptes scabiei* infestations**. *Veterinary parasitology* 2018, **251**:119-124.

172. Löwenstein M, Kahlbacher H, Peschke R: **On the substantial variation in serological responses in pigs to Sarcoptes scabiei var. suis using different commercially available indirect enzyme-linked immunosorbent assays**. *Parasitology research* 2004, **94**(1):24-30.

173. Marx V: **PCR heads into the field**. Nature Publishing Group; 2015.

174. Zhang S, Ravelonandro M, Russell P, McOwen N, Briard P, Bohannon S, Vrient A: **Rapid diagnostic detection of plum pox virus in Prunus plants by isothermal AmplifyRP® using reverse transcription-recombinase polymerase amplification**. *Journal of virological methods* 2014, **207**:114-120.

175. Lucchi NW, Gaye M, Goldman IF, Ljolje D, Deme AB, Badiane A, Ndiaye YD, Barnwell JW, Udhayakumar V, Ndiaye D: **Evaluation of the Illumigene Malaria LAMP: A Robust Molecular Diagnostic Tool for Malaria Parasites**. *Scientific Reports* 2016, **6**:36808.

176. Kong Q-M, Lu S-H, Tong Q-B, Lou D, Chen R, Zheng B, Kumagai T, Wen L-Y, Ohta N, Zhou X-N: **Loop-mediated isothermal amplification (LAMP): early detection of *Toxoplasma gondii* infection in mice**. *Parasites & Vectors* 2012, **5**(1):2.

177. Adams ER, Schoone GJ, El Safi S, Schallig HD: **Development of a reverse transcriptase loop-mediated isothermal amplification (LAMP) assay for the sensitive detection of *Leishmania* parasites in clinical samples**. *The American Journal of Tropical Medicine and Hygiene* 2010, **82**(4):591-596.

178. Jelocnik M, Islam MM, Madden D, Jenkins C, Branley J, Carver S, Polkinghorne A: **Development and evaluation of rapid novel isothermal amplification assays for important veterinary pathogens: *Chlamydia psittaci* and *Chlamydia pecorum***. *PeerJ* 2017, **5**:e3799.

179. Saito R, Misawa Y, Moriya K, Koike K, Ubukata K, Okamura N: **Development and evaluation of a loop-mediated isothermal amplification assay for rapid detection of *Mycoplasma pneumoniae***. *Journal of medical microbiology* 2005, **54**(11):1037-1041.

180. McKenna JP, Cox C, Fairley DJ, Burke R, Shields MD, Watt A, Coyle PV: **Loop-mediated isothermal amplification assay for rapid detection of *Streptococcus agalactiae* (group B *Streptococcus*) in vaginal swabs–a proof of concept study**. *Journal of medical microbiology* 2017, **66**(3):294-300.

181. Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden TL: **NCBI BLAST: a better web interface**. *Nucleic acids research* 2008, **36**(suppl_2):W5-W9.

182. Owczarzy R, Tataurov AV, Wu Y, Manthey JA, McQuisten KA, Almabrazi HG, Pedersen KF, Lin Y, Garretson J, McEntaggart NO: **IDT SciTools: a suite for analysis and design of nucleic acid oligomers**. *Nucleic acids research* 2008, **36**(suppl_2):W163-W169.

183. **Epitools epidemiological calculators.** [http://epitools.ausvet.com.au.]

184. Faber WR, Oskam L, van Gool T, Kroong NC, Knegt-Junk KJ, Hofwegenf H, van der Wal AC, Kager PA: **Value of diagnostic techniques for cutaneous leishmaniasis**. *Journal of the American Academy of Dermatology* 2003, **49**(1):70-74.

185. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T: **Loop-mediated isothermal amplification of DNA**. *Nucleic Acids Research* 2000, **28**(12):e63-e63.

186. Currie BJ, Harumal P, McKinnon M, Walton SF: **First documentation of in vivo and in vitro ivermectin resistance in *Sarcoptes scabiei***. *Clinical Infectious Diseases* 2004, **39**(1):e8-e12.

187. Walton SF, Beroukas D, Roberts-Thomson P, Currie B: **New insights into disease pathogenesis in crusted (Norwegian) scabies: the skin immune response in crusted scabies**. *British Journal of Dermatology* 2008, **158**(6):1247-1255.

188. Rampton M, Walton SF, Holt DC, Pasay C, Kelly A, Currie BJ, McCarthy JS, Mounsey KE: **Antibody responses to *Sarcoptes scabiei* apolipoprotein in a porcine model: relevance to immunodiagnosis of recent infection**. *PLoS One* 2013, **8**(6):e65354.

189. Skerratt LF, Skerratt JH, Martin R, Handasyde K: **The effects of sarcoptic mange on the behaviour of wild common wombats (*Vombatus ursinus*)**. *Australian Journal of Zoology* 2004, **52**(3):331-339.

190. Oleaga A, Alasaad S, Rossi L, Casais R, Vicente J, Maione S, Soriguer RC, Gortázar C: **Genetic epidemiology of *Sarcoptes scabiei* in the Iberian wolf in Asturias, Spain**. *Veterinary parasitology* 2013, **196**(3-4):453-459.

191. Alasaad S, Soglia D, Sarasa M, Soriguer RC, Pérez JM, Granados JE, Rasero R, Zhu X-Q, Rossi L: **Skin-scale genetic structure of *Sarcoptes scabiei* populations from**

individual hosts: empirical evidence from Iberian ibex-derived mites.

Parasitology Research 2008, **104**(1):101-105.

192. Caraco T, Cizauskas CA, Wang N: **Environmentally transmitted parasites: Host-jumping in a heterogeneous environment**. *Journal of Theoretical Biology* 2016, **397**:33-42.